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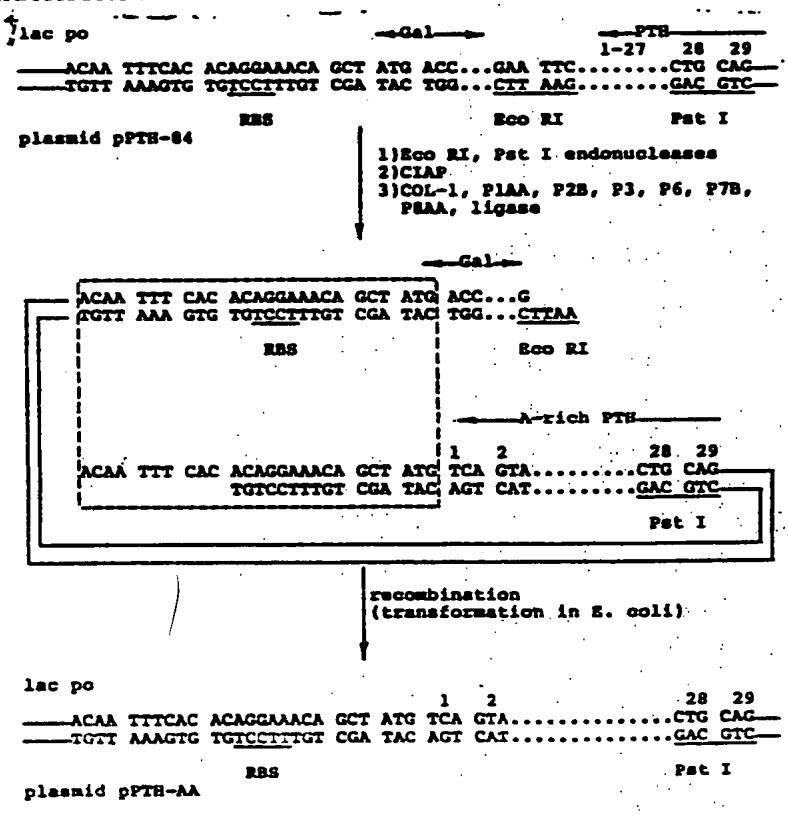
(54) Title: SYNTHESIS OF MATURE HUMAN PARATHYROID HORMONE

(57) Abstract

A nucleotide coding sequence for human parathyroid hormone has been synthesized, which sequence results in a substantially higher yield of mature PTH than hitherto could be obtained. This is achieved by providing a synthesized nucleotide sequence coding for mature human PTH or a biologically active analog, wherein the amino terminal coding sequence is adenine-rich. A sequence wherein the degenerate codons for some or all of amino acids 1 through 5 are adenine-rich is preferred.



SYNTHESIS OF PLASMID pPTH-AA CONTAINING AN A-RICH PTH-CODING SEQUENCE

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SYNTHESIS OF MATURE HUMAN PARATHYROID HORMONE

Human parathyroid hormone (PTH) is a polypeptide of 84 amino acid residues. Its amino acid sequence has been known since the 1970's (Habener 1978).

Parathyroid hormone is known to be a major regulator of blood calcium concentration (Keutmann 1974 and Keutmann 1978). Moderate doses of the hormone are known to increase bone mass (Kalu 1970). Limited clinical trials have demonstrated that the amino-terminal portion of the molecule is active in producing the characteristic effects of parathyroid hormone on bone (Habener 1978 and Reeve 1980).

PTH has, accordingly, been demonstrated as having a useful therapeutic effect in respect of bone disorders including osteoporosis and other conditions relating to bone repair and bone loss.

A 38 amino acid PTH fragment and the chemical means for its synthesis is described by R.D. Hesch in German Offenlegungsschrift 3,243,258.

A synthetic fragment consisting of the first 34 amino acids of human parathyroid hormone (hPTH-(1-34)) has demonstrated a catabolic effect in patients afflicted by osteoporosis (Reeve 1980). Bone formation rates increased markedly following long term daily subcutaneous injections in osteoporetic patients of a preparation including human PTH-(1-34) (Reeve 1980 and Reeve 1981). It is believed that parathyroid hormone stimulates bone formation indirectly, by stimulating the local production and release of a growth factor or factors within the bone. It is further believed that parathyroid hormone also stimulates osteoclasts to secrete proteases and other factors which enable osteoclasts to resorb bone, stimulate the proliferation of osteoclast precursors in bone, and increase the number of osteoclasts in bone.

While the synthetic fragment PTH-(1-34) has exhibited most of the biological activity, recent

experiments indicated discrete binding sites for the carboxyl terminal (53-84) region in renal and skeletal tissue (Murray 1989), to provide structural elements required for optimal presentation of the biologically active domain of PTH to the receptor (Born 1988). Therefore mature (i.e. intact) PTH-(1-84) may have an advantage over the shorter synthetic PTH-(1-34). Synthesis of mature PTH-(1-84) would enable such studies.

The chemical synthesis of PTH and PTH fragments is expensive and laborious. Recombinant DNA techniques provide an alternative approach. The cDNA of human preproparathyroid hormone (prepro PTH), a precursor of PTH has been cloned and sequenced (Hendy 1981). PTH was produced and secreted at 0.0015 mg/litre by inserting the precursor prepro PTH cDNA into a recombinant retrovirus for infection of rat pituitary cells (Hellerman 1984). However this yield from mammalian pituitary cells was too low to be useful.

Prepro PTH cDNA has also been cloned in Escherichia coli (Born 1987b and Born 1988). However instead of mature PTH, only shorter fragments particularly PTH-(3-84) and PTH-(8-84) were produced intracellularly. Efforts to produce or secrete mature PTH using prepro PTH cDNA in yeast has also failed (Born 1987a).

Direct expression of cDNA encoding mature PTH has been described under the control of various promoters (lac, trp or tac). E. coli transformed with mature PTH cDNA was induced to produce PTH intracellularly at a reported yield of about 0.2 mg/litre (Breyel 1984). Subsequent related studies resulted in reports of an improved production yield of PTH by E. coli to 0.47 mg/litre, through further gene manipulation (Morelle 1988). Other laboratories including our own have obtained the same order of efficiency of production of PTH by E. coli (Rabbani 1988). However on analysis of the PTH mixture, we typically isolated shorter fragments such as PTH-(8-84) (Rabbani 1988). The yield of mature (i.e. intact) PTH may accordingly be even lower than

predicted by Breyel and Morelle. Efforts to improve these relatively low yields in E. coli have been unsuccessful, and it was postulated that this was due at least in part to instability of PTH and related messenger RNA intracellularly (Morelle 1988 and Wingender 1989).

For the estimation of PTH production yield, the most commonly used method has been the Mid-Molecule PTH Radioimmunoassay, which uses an antibody specific to the narrow mid-section (44-68) of the PTH molecule. Obviously such antibody (and therefore such assay) cannot distinguish the intact PTH molecule from any degraded short fragments still maintaining the (44-68) sequence. This PTH radioimmunoassay (PTH RIA) is accordingly capable of inflating the estimation of intact PTH. Data from the Mid-Molecule PTH RIA represents merely an estimation of an immunoreactive component in the PTH mixture, but not necessarily of biologically active, intact PTH. Such shortcomings show the inadequacy of relying solely on PTH RIA for estimation and characterization of intact PTH, as is the case in some reports (Breyel and Morelle).

This detection inadequacy is evident in the expression of cDNA for mature PTH in yeast with the yeast alpha-factor prepro sequence used as the secretory signal (Gabrielsen 1988; Gautvik 1989a). Production of an immunoreactive PTH substance was estimated by these researchers as being from 1-7 mg/litre. In the PTH mixture, in addition to intact mature PTH (comprising less than 25% of the immunoreactive mixture), several fragments of PTH were also isolated, being results of in vivo cleavage at amino acid positions 27, 35 and 45 in the PTH sequence (Gabrielsen 1988). This is consistent with our own unpublished studies involving expression and secretion of alpha-factor PTH fusion protein in yeast. Our initial Mid-molecule PTH RIA detected a relatively high level of PTH, namely 20mg/litre, however, upon measuring by the more accurate Allegro PTH Radioimmunoassay (Nichols Institute

Diagnostics, San Juan Capistrano, Ca.), we discovered that less than 1% of the product could be classified as potentially intact. The Allegro PTH RIA is a two-site immunoradiometric assay simultaneously binding 2 different antibodies, one in N-terminal PTH-(1-34) sequence, the other in the mid to C-terminal PTH-(39-84) sequence. This assay accordingly provides a more accurate assessment of the yield of biologically active intact PTH than does a Mid-molecule assay used by, for example, Gabrielsen, which merely identifies a single mid-molecule binding site and therefore could conceivably be measuring an immunoreactive PTH mixture containing PTH fragments in addition to intact PTH.

In another study, cDNA encoding mature PTH was cloned in E. coli, and PTH is reported as having been produced as a secreted polypeptide at a yield up to 10 mg/litre (estimated by Mid-molecule PTH RIA using a protein A leader sequence and promotor (Gautvik 1989a). However intact PTH constituted less than one-third of the immunoreactive PTH mixture (Gautvik 1989b). Unpublished work in our laboratory using a secretory system of E. coli for production of PTH was able to achieve a yield of 15 mg/litre of PTH (when measured by Mid-molecule PTH RIA), but only at most 10% of this product mixture comprised potentially intact PTH (when measured by Allegro PTH RIA).

Because of the recognized problem of protein instability, it has also been attempted to express PTH as a fusion protein with beta-galactosidase which results in a measured yield of 35-50 mg of PTH/litre (Wingender 1989). The fusion site Asp-Pro permits a chemical cleavage by acid hydrolysis to yield an 85 amino acid-proline-PTH analog. However, there is no established method for the removal of the extra proline in order to generate authentic intact PTH.

In the result, all known recombinant means of expressing mature human PTH have resulted in low production yields, and prevalence of PTH fragments or analogs, many of which are not biologically active. Where the expression

vehicle is E. coli, and PTH is produced as a secreted polypeptide, there is an adverse effect on yield due to proteolytic degradation during secretion.

We have now synthesized a nucleotide coding sequence for human parathyroid hormone, which sequence results in a substantially higher yield of mature PTH than hitherto could be obtained. The nucleotide sequence encoding the amino-terminal end of PTH, particularly amino acids 1 through 28, especially amino acids 1 through 5, have been synthesized for optimal expression of intact human PTH. We have established the conditions for optimizing the expression of our synthesized human PTH gene.

It is a feature of this invention to provide a means for the synthesis of mature human PTH including biologically active analogs, which avoid the low yields and product instability problems of the prior art methods.

It is another feature of this invention to provide a synthesized nucleotide coding sequence, which codes for mature human PTH including biologically active analogs.

It is another feature of this invention to provide an expression system for intracellular production of mature human PTH and biologically active analogs.

It is another feature of this invention to provide a method of expressing and recovering mature human PTH and biologically active analogs.

It is another feature of this invention to provide transformed cells containing synthetic nucleotide sequences encoding mature human PTH and biologically active analogs.

The invention achieves these features by providing a synthesized nucleotide sequence coding for mature human PTH or a biologically active analog, wherein the amino terminal coding sequence is adenine-rich. A sequence wherein the degenerate codons for at least some of

amino acids 1 through 5 are adenine-rich is preferred. A sequence wherein the degenerate codon for amino acid 1 is guanine-rich and the degenerate codons for amino acids 2 and 3 are adenine-rich, and a sequence wherein the degenerate codons for amino acids 1 through 3 are adenine-rich are particularly preferred. It is also preferred to select degenerate codons for the amino terminal coding sequence so as to avoid constituting a potential ribosome-binding site. Also preferred is any of the above sequences modified to code for a mutant PTH wherein aspartic acid is substituted for the usual amino acid at position 38.

In drawings illustrating embodiments of the invention,

Figure 1 is a schematic diagram summarizing the synthesized nucleotide sequence encoding mature human PTH contained in plasmid pPTH-84;

Figure 2 is a schematic diagram showing the two step synthesis of plasmid pPTH-84;

Figure 3 summarizes the synthesized nucleotide sequence encoding PTH-(1-28) in adenine-rich plasmid pPTH-AA;

Figure 4 is a schematic diagram summarizing the construction of plasmid pPTH-AA;

Figure 5 summarizes the synthesis of adenine-rich plasmid pPTH-AA;

Figure 6 summarizes the synthesized nucleotide sequence encoding PTH-(29-84) constructed with degenerate codons in the usage frequency favoured by E. coli;

Figure 7 is a schematic diagram summarizing the construction of plasmid pPTH-AA-Eco;

Figure 8 is a depiction of the gel electrophoretic analysis of product expressed by various synthesized PTH plasmids;

Figure 9 is a depiction of immunological blot identification of intact PTH and short analog PTH-(8-84) produced in E. coli;

Figure 10 is a depiction of immunological blot identification of intact PTH and short analog PTH-(8-84) produced from plasmids containing human-favoured codons at the amino terminal of the PTH gene;

Figure 11 is HPLC chromatograms illustrating the purification of recombinant PTH;

Figure 12 is mass spectra of purified recombinant PTH-(1-84) and PTH-(8-84); and

Figure 13 is a graph of an adenylate cyclase assay of recombinant intact PTH.

Taking advantage of the known degenerate codons for amino acids, we synthesized various plasmids each containing a PTH-coding nucleotide sequence rich in adenine, particularly at codons relating to amino acids 1 through 28, and especially at one or more of the codons relating to amino acids 1 through 5. We discovered that an adenine-rich amino-terminal coding sequence resulted in the selective production of mature human PTH at a substantially higher yield. In particular, when amino acids 1 through 5 of PTH were coded in an adenine-rich fashion, yields of mature PTH were increased by a factor at least of 10 over that obtained using normal human PTH codons for amino acids 1 through 5.

We have also discovered that fragments of PTH, such as PTH-(8-84) become the major component of the PTH product whenever the codon ATG encoding the amino acid methionine at position eight of PTH, functions as an internal start codon. Normally, codon ATG immediately upstream of the PTH coding sequence codes for formyl methionine, and should serve as a starting codon for PTH, with the formyl methionine being excised in the normal course of events once the PTH has been synthesized. However, with the utilization of any guanine-rich

degenerate codons especially at positions 3 and/or 4, ATG at position eight is efficiently misread as though it were the start codon, thus bypassing the upstream ATG, and causes mainly synthesis of PTH-(8-84), thereby greatly reducing the total yield of intact PTH. The PTH fragment produced is (8-84) rather than (9-84) because methionine at position eight is not successfully removed from its neighbouring amino acid, histidine, at position nine, histidine having (compared with serine at position one) a relatively large radius of gyration which prevents the usual methionine aminopeptidase system from operating. (Sherman 1985).

The following examples provide a more detailed description of the invention.

PRELIMINARY EXAMPLE: Preparation of plasmids pPTH-84 and pPTH-84c containing synthesized nucleotide sequence.

We earlier synthesized a nucleotide sequence encoding the known amino acid structure for human PTH and synthesized plasmids containing our synthesized nucleotide sequence. (Sung 1986a and Rabbani 1988). These procedures are set out below. The synthesized nucleotide sequence encoding human PTH in plasmid pPTH-84 is set out in Figure 1. The nucleotide sequence generally utilizes degenerate codons in the frequency favoured by yeast, for the amino acids encoded.

Materials and methods

Enzymes and plasmid pUC8 were purchased from Bethesda Research Laboratories and Boehringer Mannheim. *Escherichia coli* K-12 strain JM103 (Δ(*lac pro*), *thi*, *str* A, *sup* E, *end* A, *sbc* B, *hsd* R, F *tra* D36, *pro* AB, *lac* I^{Q}, ZΔM15) was used.

Synthesis of ologonucleotides

The sixteen deoxyribooligonucleotides PI-PXVI (Figure 2), encoding PTH with the frequently used yeast

codons, were synthesized by DNA synthesizer model 380A (Applied Biosystem) and purified on 12% polyacrylamide gel containing 7 M urea.

Construction of plasmid pPTH-34 and pPTH-40

Each of the eight oligonucleotides PI-PVIII (1.3 pmol, 1 μL) (A in Figure 2) was phosphorylated in a mixture containing 0.4 μL of 10 X kinase buffer, 0.4 μL of 1 mM ATP, 0.4 μL of T4 DNA kinase, and 3 μL of water. Phosphorylation reaction was carried out for 1 h at 37°C. The solutions were then combined and heated to 70°C for 10 min. After being cooled slowly to room temperature, the combined solutions were added to a mixture of 3.5 μL of 10 X ligase buffer, 3.5 μL of 4 mM ATP, 0.1 pmol of EcoRI-HindIII linearized plasmid pUC8, and 3.5 μL of T4 DNA ligase and incubated at 12°C for 20 h. Aliquots of the ligation mixture were used to transform E. coli JM103 in YT plate containing ampicillin. Transformants were selected by the loss of β-galactosidase activity (X-Gal and isopropylthiogalactoside) for hybridization analysis.

Labelling of the hybrization probe

Oligonucleotides PI-PV (10 pmol, 1 μL) were phosphorylated individually with [^{32}P]ATP (10 pmol, 3 μL) in 1 μL of T4 DNA kinase, 1 μL of 10 X kinase buffer, and 4 μL of water at 37°C for 1 h.

Screening of plasmid containing the PTH-(1-40) gene

Colonies were chosen and grown on 10 copies of nitrocellulose filters on YT plates with ampicillin overnight. They were then denatured with 0.5 N NaOH-1.5 M NaCl (10 min) and neutralized with 0.5 N Tris-HCl (pH 7.0) - 1.5 M NaCl (10 min). After 2 h at 80°C in a vacuum oven, the filters were washed with 6 X SSC - 0.05% Triton X-100 for 30 min. Cell debris was scraped off completely. After another 30 min in fresh solution, the duplicate filters were transferred individually into separate

mixtures of 6 x SSC - 1% dextran sulphate - 0.05% Triton X-100 - 1 Denhardt's hybridization fluid. Individually appropriate ^{32}P-labelled probes were added to a pair of filters. After 16h at 45°C, filters were washed twice with 6 x SSC - 0.05% Triton X-100 at room temperature for 5 min and then at 45°C for 45 min, and were analyzed by autoradiography. Filters were washed again at 75°C for 45 min, followed by autoradiographic analysis.

Preparation of plasmids pPTH-34 and pPTH-40 via sub-cloning

Transformants positively identified by either probe PIV or PV were cultured for the minipreparation of plasmids to transform the E. coli JM103 once again. Colony hybridization with ^{32}P-labelled probes PVI and PV were used to identify plasmid clones, pPTH-40 encoding the regular amino acid sequence from position 1 to 40 of PTH and pPTH-34 which has termination at oligonucleotide triplet position 35. The PTH-coding region was sequenced with the dideoxytermination method.

Construction of plasmids pPTH-84 and pPTH-87

Plasmid pPTH-40 was linearized by incubating with restriction enzymes SstI and HindIII. The phosphorylation of the other eight oligonucleotides PIX-PXVI (B in Figure 2) and their ligation into the linearized plasmid pPTH-40 were similar to the construction of the latter plasmid. Transformed JM103, with plasmids bearing the whole PTH gene, was identified by hybridization with ^{32}P-labelled probes PIX-PXIII. Isolated plasmids were similarly subcloned and analyzed by DNA sequencing with the "dideoxy" method.

Results

Eight synthetic oligonucleotides PI-PVIII, constituting the first half (oligonucleotide triplet positions 1-40) of the PTH gene (A in Figure 2), were

phosphorylated and ligated directly into linearized plasmid pUC8 in a single operation without any intermediate purification of gene assembly.

Base mismatch was designed at nucleotide triplet position 35, between complementary oligonucleotides PIV (GTT, valine) and PV (TTA, complementary triplet of the termination codon) (A in Figure 2). Transformation in JM103 by recombinant plasmid-bearing fragments PI-PVIII subsequently yielded two plasmids: pPTH-40 coding for a legitimate half of PTH (PTH 1-40) with the termination codon in the HindIII site and pPTH-34 encoding a shorter fragment (PTH(1-34)) because of the predetermined termination codon at triplet position 35.

Hybridization with ^{32}P-labelled PI-PV at 45°C identified transformants bearing the general PTH-coding sequence. At an elevated temperature of 75°C, both PIV and PV were capable of distinguishing betwen colonies predominant with plasmids pPTH-40, and pPTH-34, respectively. DNA sequencing of the two plasmids confirmed that pPTH-40 had a valine codon (complementary triplet AAC) at position 35 and pPTH-34 had termination (complementary triplet TTA) at the same site.

Plasmid pPTH-40 was then digested with restriction endonucleases SstI and HindIII. Synthesis of the whole PTH gene was then completed via phosphorylation and ligation of another eight synthetic oligonucleotides, PIX-PXVI constituting the rest of the PTH gene (positions 39-84) (B in Figure 2), into the linearized plasmid pPTH-40.

Base mismatch at oligonucleotides triplet position 85 of the two complementary fragments PXII (TAA, termination codon) and PXIII (ACA, complementary triplet of cysteine) resulted in the formation of two different PTH gene-bearing plasmids, pPTH-84 and pPTH-87. These bore the proper coding sequence with termination at position 85 or a cysteine codon (TGT) at the same position, respectively.

Bacterial transformants with these plasmids were identified by hybridization with ^{32}P-labelled fragments

PIX-PXIII as before. Plasmids were prepared from these transformants for subcloning. Dideoxy DNA sequencing of the subcloned plasmids showed two types of PTH-coding plasmids pPTH-84 and pPTH-87, with their difference only at position 85 of the PTH gene. Plasmid pPTH-84 has the termination triplet codon at this position and plasmid pPTH-87 has cysteine codon.

Another expression plasmid pPTH-84c was constructed by digesting pPTH-84 with EcoRI and PstI to remove the 5' portion of the PTH gene. The large restriction fragment bounded by EcoRI and PstI sites were isolated and litigated with six synthetic overlapping oligonucleotides which reconstructed the 5' end of the PTH gene with an ATG starting codon at amino acid-1. A crossover linker sequence (Sung 1986b) was designed at the upstream end, which was homologous to the sequence encompassing the ribosomal binding site to the starting ATG of the β-galatosidase gene already present in the opposite terminus of the plasmid intermediate. After transformation of E. coli JM103, the homologous termini recombined in vivo to yield plasmid pPTH-84c. The new plasmid pPTH-84c was present in 4% of all transformants. The construction of the plasmid in the region of the ribosomal binding site and PTH coding region was confirmed by nucleotide sequencing.

EXAMPLE 1: Synthesis of human PTH-coding nucleotide sequence with their PTH(1-5) domain rich in adenine, guanine, cytosine or thymine.

Using plasmid pPTH-84, we designed and synthesized various PTH nucleotide coding sequences in two separate segments, namely PTH-(1-28) and PTH-(29-84). As nucleotide coding sequence for the segment PTH-(29-84), namely the mid to C-terminal region, we used the abovementioned sequence constructed with yeast-favored codons, described by us previously in plasmid pPTH-84.

Using standard published protocols, the abovementioned precursor plasmid pPTH-84 previously

described by us (see Preliminary Example), was linearized by endonucleases PstI and EcoRI (Maniatis 1982). The PstI/EcoRI-linearized plasmid containing the PTH-(29-84) sequence was utilized for the construction of new PTH genes with different oligonucleotide contents at the amino terminus, as referred to below. The linearized plasmid was dephosphorylated with calf intestine alkaline phosphatase (CIAP) according to Maniatis. The PstI/EcoRI/CIAP-treated plasmid pPTH-84 was used directly for the construction of novel plasmids.

Various coding sequences of the segment PTH-(1-28) were constructed. These were designed so as to demonstrate the effect of varying available degenerate codons while at the same time encoding the normal human PTH amino acid sequence at positions 1 through 28. In order to achieve this, a series of oligonucleotides encoding amino acids 1 through 8 of PTH were designed using the various degenerate codons available for the specified amino acids. Each oligonucleotide was selected so as to have the maximum number of adenine (A) molecules in the first five codons, as well as, for comparative purposes, the maximum number of each of cytosine (C), guanine (G) or thymine (T) in the first 5 codons. Thus the N-terminal amino acid (1-5) sequence of PTH namely (methionine)-serine-valine-serine-glutamic acid-isoleucine... could be encoded by the various synthesized nucleotide sequences illustrated in Table 1.

In the result, we synthesized a series of oligonucleotides encoding PTH-(1-8), namely P1AA, P1CC, P1GG, and P1TT, rich in each of the specific nucleotides A, C, G and T, at coding positions 1 through 5.

Complementary oligonucleotides encoding PTH-(1-8), namely P8AA, P8CC, P8GG and P8TT, were also synthesized with each possessing a homology-searching sequencing for subsequent integration with the ribosome-binding sequence in the plasmid.

An oligonucleotide duplex (P2B/P7B) was designed to encode PTH-(9-17). In this example, that oligonucleotide duplex was not designed with any effort to make it rich in a specific nucleotide. A further oligonucleotide duplex (P3/P6) was similarly designed to encode PTH-(18-28). These synthetic oligonucleotides encoding PTH-(1-8), PTH-(9-17) and PTH-(18-28) together reconstruct the amino end (1-28) of the human PTH gene. A cross-over linker sequence COL-1 is designed at the amino end which is homologous to the abovementioned complementary sequence encompassing the ribosome-binding site for the starting codon already present in the plasmid pPTH-84. The two homologous termini are capable of recombining in vivo to obtain plasmid pPTH-AA, and in similar fashion, pPTH-CC, pPTH-GG, and pPTH-TT as described below.

This procedure is illustrated for pPTH-AA in Figures 3, 4 and 5. In Figure 3, the nucleotide sequence of the synthesized (1-5) adenine-rich oligonucleotide duplexes is set out. The construction of plasmid pPTH-AA therefrom is illustrated in Figures 4 and 5. With reference to Figures 3, 4 and 5, overlapping oligonucleotide duplexes COL-1, P1AA, P2B, P3, P6, P7B, P8AA (hatched in Figure 4), constituting the coding sequence of PTH-(1-28), were ligated to the PstI end of the linearized plasmid pPTH-84. The homology-searching sequence of the COL-1/P8AA duplex (black in Figure 4) recombines with the identical ribosome-binding site (black) of the gal gene at the opposite plasmid terminus in vivo during transformation of E. coli. The plasmid was circularized to yield new plasmid pPTH-AA with the residual gal gene (stippled in Figure 4) deleted.

In summary, the constructed A-, C-, G- or T-rich oligonucleotides encoding amino acids 1 through 8 were, separately, phosphorylated together with the cross-over linker COL-1, the oligonucleotide encoding amino acids 9 through 17, and the oligonucleotide encoding amino acids 18 through 28. The phosphorylation solution contained 0.23 mM

ATP, 70 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 100 mM KCl, 5 mM DTT and 30 U of T_4 DNA kinase at 37°C for 1.6 hr. The complementary oligonucleotides were phosphorylated under identical conditions. The two phosphorylation solutions were combined and heated at 80°C for 12 minutes. The combined solution was cooled slowly to 22°C in a water bath to ensure annealing of the oligonucleotides. This mixture was added to a 7 μl solution containing 75 mM Tris-HCl pH 7.5, 7.5 mM $MgCl_2$, 12.5 mM DTT, 1.2 mM ATP, 2 U of T_4 DNA ligase and 50 ng (0.025 pmol) of the PstI/EcoRI/CIAP-treated plasmid pPTH-84. After incubation at 12°C for 16 hours, this ligation mixture was used to transform E. coli JM103 on YT plates (8g Bacto-Tryptone, 5g Bacto-Yeast Extract, 5g NaCl, 15g Bacto-Agar all in 1 litre) containing 100 μg ampicillin/ml, following the protocol described by Maniatis. Transformants were regrown on nylon filters (Magna, MSI) on YT ampicillin plates for 16 hours. The colonies were denatured with 0.5 M NaOH-1.5 M NaCl (10 mins.) and neutralized with 0.5 M Tris- HCl (pH 7.0) - 1.5 M NaCl (10 mins.). After 2 hrs. at 80°C in a vacuum oven, the filters were washed for 30 mins. with SSC soln. which was made of sodium chloride (52 g/L), sodium citrate (29 g/L) and 0.05% Triton X-100. Cell debris was removed completely from the filter. The filter was placed into the 10 mL of SSC solution, which also contained 1% dextran sulphate, Ficoll (100 mg/L), polyvinylpyrrolidone (100 mg/L) and bovine serum albumin (Fraction V, 100 mg/L).

For the construction of plasmid pPTH-AA, the ^{32}P-labelled probe P8AA (10 pmol) was used to identify clones containing the new plasmid pPTH-AA. After 16 hrs. at 45°C, the filter was washed with SSC solution at 65°C for 30 mins., followed by autoradiographic analysis. Of 425 transformants tested, 61 retained affinity to the labelled probe P8AA (mutant population 14%) and were selected for preparation of plasmids. Nucleotide sequencing by the well-established dideoxytermination method confirmed the construction of plasmid pPTH-AA which possessed an A-rich

N-terminal coding sequence properly integrated to the ribosome-binding site of, in this case, the lac promotor.

Other plasmids such as pPTH-TT, pPTH-CC, and pPTH-GG were also constructed with the same protocol. For generating the maximum number of a specific nucleotide (C, G or T) in the PTH-(1-5) region, appropriate oligonucleotide duplexes encoding the PTH-(1-8) region were used instead of the P1AA/P8AA duplex.

Each transformant was cultured in 5 ml of a 2YT medium (16 g Bacto-Tryptone, 10 g Bacto-Yeast Extract and 10 g NaCl in a litre) additionally containing ampicillin (100 mg/L) and isopropyl β-D-thiogalactoside (final concentration 0.7 mM) at 37°C for 10 hours. Cells were harvested after centrifugation. A 1% SDS solution (1.25 ml) was added. The cells were lysed by sonication. The PTH content was estimated by the 2-site Allegro PTH Radioimmunoassay following the manufacturer's instructions.

A comparison of the yields of the transformants produced using each of the constructed plasmids revealed that the A-rich N-terminal sequence in plasmid pPTH-AA resulted in a dramatic increase in yield of immunoreactive PTH, for example when compared with the yield of plasmid pPTH-84c, as illustrated in Table 1. These comparisons show that substantially higher yields of PTH are obtained using plasmids rich in adenine at nucleotide codons encoding amino acids 1 through 5, when compared with other PTH plasmids under the control of the same promoter.

Both plasmids pPTH-CC and pPTH-TT, with PTH-(1-5) coding sequences rich in C and T, generate PTH with low efficiency consistent with those previously described by Breyel and Rabbani, despite the fact that in the case of pPTH-TT the five codons (TCT-GTT-TCT-GAA-ATA) encoding the PTH-(1-5) region are so-called E. coli-favoured codons (Grantham 1980). The G-rich N-terminal sequence of plasmid pPTH-GG initially appeared to generate a higher yield of an immunoreactive PTH substance (estimated by Allegro RIA),

however, further studies revealed that the product included a large proportion of biologically inactive PTH fragments namely PTH-(8-84).

Since the first and third amino acid residues in PTH are serine, six different degenerate codons are available for coding it. Plasmid pPTH-CompB was constructed in the same fashion as above described using the AG-containing codon for serine as opposed to the TC-containing codon used to construct plasmids pPTH-AA, -CC, -GG and -TT (see Table 1). Using this codon, yield of intact PTH was generally improved (see Table 1), although lower than when using plasmid pPTH-AA.

EXAMPLE 2: Synthesis of human PTH-coding nucleotide sequences with PTH-(1-28) domain rich in adenine and plasmids containing them.

We next sought to establish whether there would be any effect of enhanced adenine richness at sites other than those coding for amino acids 1 through 5. Using the same procedure as already described, and plasmid pPTH-84 synthesized using yeast-favoured codons, we constructed plasmids pPTH-wA and pPTH-wxA containing additional adenine richness at various codons between positions 12 and 28, as illustrated in Table 1. As set out in Table 1, the estimated PTH yield using pPTH-wA and pPTH-wxA was a similar order to that obtained using pPTH-AA. It will be noted that in both cases, there was no improvement in yields of human PTH over that obtained by plasmid pPTH-AA. This suggests that only adenine immediately downstream from the starting codon ATG exerts the noted positive effect on expression.

EXAMPLE 3: Effect of adenine richness at various of the (1-5) codons.

Using the same methods, we also constructed plasmid pPTH-hA, with its PTH-(1-5) codons identical to those of human PTH cDNA (i.e. (ATG)-TCT-GTG-

AGT-GAA-ATA...). The nucleotide sequence for the N-terminal end of pPTH-hA is indicated in Table 1. Codons 3 to 5 but not 1 and 2 are adenine-rich. This plasmid resulted in a relatively low yield of PTH. This suggests that adenine richness in codons 1 and 2 is important in obtaining the improved yield of intact PTH according to this invention.

EXAMPLE 4: Effect of E. coli-favoured degenerate codons dominating in PTH-(29-84).

Plasmid pPTH-AA-Eco was also constructed using nucleotide sequence dominated by E. coli-favoured degenerate codons for encoding the mid through C-terminal regions PTH-(29-84) (Chen 1982). By the procedure previously described, plasmid pPTH-AA was linearized using endonucleases PstI and HindIII. Oligonucleotides P103b, P104, P105, P106, P201, P202, P203 and P204a constituting a PTH-(29-84) coding sequence adapted predominantly from degenerate codons in the usage frequency favoured by E. coli, as described in Figure 6 were ligated into the PstI/HindIII-treated plasmid as illustrated in Figure 7. The new plasmid, pPTH-AA-Eco was, when expressed in a transformed host, capable of somewhat higher yields (5.5 mg/L as shown in Table 5) in E. coli JM103 under the induction of isopropylthiogalactoside (IPTG) than plasmid pPTH-AA which possessed yeast-favoured frequency of codon usage in PTH-(29-84) coding sequence.

We then carried out the same experiment by constructing two plasmids pPTH-AA-Eco(18-84) and pPTH-AA-Eco(8-84) with the same codons (1-5) as pPTH-AA-Eco, but with E. coli-favoured codons encoding PTH-(18-84) and PTH-(8-84) regions respectively. The former obtained substantially the same yield of intact PTH in transformed E. coli strain JM103 as plasmid pPTH-AA-Eco, while the latter had a substantially lower yield.

Table 2 summarizes the structure of various synthesized plasmids which possess identical PTH-(29-84)

nucleotide sequence constituted by E. coli-favoured codons, as well as the intact PTH yield of E. coli Y1091 transformed with such plasmids.

We conclude that further extending the E. coli-favored frequency of codon usage in the coding sequence upstream from amino acids 28 to 8 of PTH has little or no positive effect on the expression of intact PTH in E. coli. Indeed substitution of the adenine rich codons in regions of the N-terminal coding sequence with codons favoured by E. coli can decrease the yield of PTH as, for example, in the case of plasmids pPTH-AA-Eco(8-84) and pPTH-TT whose T-rich codons 1 through 5 are generally those favoured by E. coli for the amino acids 1 through 5 encoded.

The same PTH-(29-84) nucleotide sequence of pPTH-hA-Eco, however, failed to improve the yield of PTH, thereby indicating an inefficiency of its N-terminal PTH-(1-5) coding sequence, which is identical to that of human cDNA.

EXAMPLE 5: Effect of internal starting codon and ribosome-binding sites on PTH expression

The analog PTH-(8-84), a byproduct of expression, can theoretically be derived via (i) proteolytic degradation of intact PTH, or (ii) internal initiation of expression at ATG-8. As in plasmids pPTH-GG, pPTH-GG-Eco, pPTH-hA-Eco and pPTH-84c which produce such analog, their codons in the PTH-(2-5) region may have constituted a ribosome-binding site to initiate competing expression at the codon ATG-8. For the confirmation of such possibility, more new plasmids were constructed, with their degenerate codons constituting various potential ribosome-binding sites at the PTH-(1-5) domain (Table 3). After expression, the ratio of intact PTH and the analog PTH-(8-84) was determined by Western immunoblot with antibodies specific to the PTH-(69-84) region. As predicted, the production of fragment PTH-(8-84) is confined to plasmids which possess a

potential ribosome-binding site (G-rich sequence interspersed by A, i.e. as descrbied in Shine 1974) in the PTH-(2-5) region, without any exception.

Among plasmids which exclusively produce intact PTH, the most efficient plasmids were pPTH-AA-Eco and pPTH-CompE-Eco, both possessing the highest adenine composition in the N-terminal coding region. However, the expression efficiency was decreased when adenine of the degenerate codons was substituted by other bases, especially with cytosine and thymine (Table 3).

Among the remaining plasmids which produce both intact PTH and PTH-(8-84), the proportion of intact PTH in the mixture also decreased with the substitution of adenine in the PTH-(1-5) domain by other bases (Table 3). The most severe cases were in plasmids pPTH-GA11-Eco and pPTH-GA12-Eco where adenine of codons 1 and 2 was substituted by cytosine and thymine. Though encoding intact PTH, plasmid pPTH-GA12-Eco produces exclusively fragment PTH-(8-84), without any trace of intact PTH detected. Apparently this is a result of two factors, (i) poor expression of intact PTH because of low adenine composition in the N-terminal region, and (ii) a strong internal ribosome-binding site favoring the expression of PTH-(8-84). Therefore, our data confirm that the nucleotide sequence encoding the PTH-(1-5) region predetermines both the potential expression of the short analog and the expression efficiency for intact pTH.

While synthetic PTH genes were used in our laboratory, all prior studies of PTH expression in E. coli by other researchers, involved the human PTH cDNA. However, the human PTH cDNA possesses a ribosome-binding site-like sequence GTG.AGT (underlined) in the PTH-(2-3) region, for the potential expression of PTH-(8-84). In the expression of the human preproPTH cDNA in E. coli, the short analog PTH-(8-84) was indeed obtained as a byproduct (Born 1987b). In another earlier study, a PTH-(1-84) nucleotide sequence (without the prepro sequence) derived

from cDNA has been used for expression in E. coli, by other laboratories (Breyel 1984). Although an immunoreactive PTH product was produced at 0.2 mg/L (estimated by PTH radio-immunoassay) under the control of a lac promotor, its identity has never been properly established. It is highly probable that the uncharacterized product of this earlier study was a mixture of intact PTH and PTH-(8-84). Such a possibility of competing expressions of intact PTH and PTH-(8-84) were further supported by the expression of our plasmids pPTH-hA-Eco and pPTH-hA. Both were constructed with the codons at the PTH-(1-5) domain identical to those of human PTH cDNA (Breyel 1984). As predicted, under the control of a lac promotor, expression yielded a 2:1 mixture of intact PTH and the analog PTH-(8-84) with a total yield of 0.2-0.3 mg/L (Tables 1, 2 and 3, Figure 10). All these results thus indicate that the cDNA-derived PTH coding sequence is undesirable for direct expression in E. coli, because of its poor expression efficiency and the competing production of PTH-(8-84). In addition, such byproduct would complicate any subsequent purification of intact PTH.

EXAMPLE 6: Selection of appropriate host cell and expression conditions.

In testing the expression of our constructed plasmids, we transformed various E. coli strains with them as illustrated in Tables 4 and 5. E. coli strain Y1091, when transformed with our plasmids, generally expressed PTH at a yield considerably greater than that of similarly transformed JM103 and HB101, in terms of mg PTH/L culture and percentage of total bacterial protein. Increased culture periods beyond 10 hours at 37°C, were, in general, not found to be an effective means of improving yields (see Tables 5 and 6). Once the optimum culture period had expired, longer culture periods generally only proved detrimental to yield. Our study of bacterial hosts used for expression of our synthesized plasmid clearly indicates

that E. coli Y1091 was consistently superior to other commonly used E. coli strains. Such improvement in PTH production by host substitution (to lon strain) contradicts the results of Breyel.

In JM103 transformants, the induction of the lac promoter was essential for PTH expression. However, induction was not needed in the lon strain Y1091. The PTH gene, though under the control of the lac promoter, does not require induction of isopropylthiogalactoside (IPTG) in Y1091 (see Table 5). The exclusion from the culture medium of isopropylthiogalactoside which is an expensive reagent would be economically beneficial to any large scale production of PTH, thus E. coli Y1091 is to be preferred for that reason as well as for its improved yield and ratio of intact PTH to total bacterial protein.

Our studies related to the establishment of culture conditions for optimal expression of PTH are summarized in Table 6. We carried out a time study of the production of PTH by E. coli Y1091 transformed by plasmid pPTH-AA-Eco. The expression was carried out at 37°C, in 2YT medium in the presence of 1% Casamino acids. Maximum yield was obtained after permitting expression intracellularly for 10 hours.

EXAMPLE 7: Analysis of expressed PTH by gel electrophoresis.

The products expressed by different E. coli Y1091 transformants were analyzed. Transformants possessing plasmids pPTH-AA-Eco and pPTH-GG-Eco and were cultured in 2YT + 1% Casamino acids, 100 mg ampicillin/L, at 37° for 10 hours. Cells harvested after centrifugation were lysed by 1% SDS. Electrophoresis of total cell lysate was carried, out on 1% SDS - 17% polyacrylamide gel (14x16 cm, 1mm) (stained by Coomassie blue). Application dosage of each sample was equivalent to 40 µl of the original culture. The results are illustrated in Figure 8, where

numbers in left margin indicate molecular weight standards.

Lane a - clone with plasmid pUC8 with no PTH gene (negative control)

Lane b - clone with plasmid pPTH-GG-Eco producing PTH-(8-84).

Lane c - clone with plasmid pPTH-AA-Eco producing intact PTH.

Lane d - clone with plasmid pUC8 plus synthetic intact PTH.

Lane e - synthetic intact PTH.

Lane f - purified recombinant PTH.

EXAMPLE 8: Immunological (Western) blot characterization of PTH expressed by E. coli.

Antibodies specific to amino and carboxyl termini of PTH were prepared as follows. PTH-(69-84) amide was synthesized on methybenzhydrylamine resin, using tBoc chemistry (Stewart 1984). PTH-(1-17) was constructed on a branched lysine core as described (Posnett 1988). The core was constructed on a phenylacetamidomethyl resin, with a β-alanyl spacer, and using bis-tBoc-Lys. Rabbit antibodies were developed directly to the PTH-(1-17)-Lys complex and to PTH-(69-84) coupled to keyhole Limpet hemocyanin. The antibodies were affinity purified by passage through a column of PTH-(1-17) coupled to AffiGel 15 or PTH-(69-84) coupled to AffiGel 10.

The protein products of different transformants were analysed by an immunoblotting procedure. Whole cell lysates of E. coli Y1091: pPTH-AA-Eco and Y1091:pUC8 were committed to electrophoresis on 1% SDS - 17% polyacrylamide gel (14x16 cm, 1mm) as described in detail in Example 8 (except using 24 μl of culture). Without staining, the protein contents were electro-transferred from the gel onto two nitrocellulose membranes which sandwiched the gel (200 mamp, 15 min. and then current reversed for 45 min.). The

membranes (Towbin 1979) were saturated with 10% fetal calf serum, and were immunoblotted separately with the anti-PTH-(69-84) antibodies (Figure 9A) and the anti-PTH-(1-17) antibodies (Figure 9B). After standard treatment in substrate solution (nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, 30°, 20 min.), dark bands could be observed, indicating PTH-related protein. The results are indicated in Figure 9, where the numbers in the left margin show molecular weight standards, as well as the positions of intact PTH and analog PTH-(8-84).

Lane a - E. coli Y1091:pUC8 (negative control)
Lane b - E. coli JM103:pPTH-GG.
Lane c - E. coli Y1091:pPTH-GG-Eco
Lane d - E. coli JM103:pPTH-AA
Lane e - E. coli Y1091:pPTH-AA-Eco.
Lane f - E. coli purified recombinant PTH
Lane g - synthetic intact PTH from Bachem Ltd.

EXAMPLE 9: Characterization of intact PTH expressed by E. coli strain Y1091, transformed by plasmid pPTH-AA-Eco.

(a) Radioimmunoassays were carried out according to known procedures. Both estimations by the Mid-molecule PTH Radioimmunoassay and the Allegro PTH Radioimmunoassay were identical, indicating all recombinant PTH was likely in the form of intact molecule.

(b) Gel electrophoresis of a lysate of clone Y1091: pPTH-AA-Eco on SDS-polyacrylamide gel (stained by Coomassie blue dye) indicated only a single new band. It has the same mobility as synthetic human PTH (manufactured by Bachem Ltd.). (See Figure 8).

(c) Western blotting with antibody specific to PTH-(1-17) as in Figure 9B or PTH-(69-84) as in Figure 9A confirmed the new polypeptide as PTH-(1-84). Short analogs, such as PTH-(8-84) previously reported (Born 1987b; Rabbani 1988), were not detected.

(d) The yield of intact PTH in this system (20 mg/L or 2.5% of total bacterial protein) is 50-100 fold better than results of Breyel and Rabbani.

Exclusive production of PTH-(1-84) was also confirmed in transformants possessing plasmids pPTH-AA, pPTH-CompB, pPTH-wA-Eco, pPTH-A-Eco(18-84), pPTH-A-Eco(8-84), and pPTH-CompE-Eco.

Our PTH gene of plasmid pPTH-AA-Eco lacks the structural features common in some highly expressed genes (Gold 1981). Sometimes the efficiency of translation of some genes can be improved by the elimination (or weakening) of secondary structure in the mRNA (Hall 1982; Tessier 1984). In the PTH mRNA of plasmids pPTH-CC and pPTH-TT, hairpin loops can potentially be formed between the A,G-rich ribosome-binding site and the amino-terminal region with respective ΔG (free energy) values of -7.0 and -9.6 kcal (Tinoco 1973), to interrupt the translation process. The A-rich degenerate codons in the PTH-(1-5) region of plasmid pPTH-AA, may weaken such secondary structure (ΔG = -3.2 kcal) and consequently improve PTH expression. However, such mechanism fails to explain the poor PTH expression by plasmid pPTH-84c, which has a similarly weakened secondary structure in its PTH mRNA (ΔG= -3.4 kcal).

In addition, five codons TCA-1, GTA-2, TCA-3, ATA-5 and TTA-7 at the PTH N-terminus of plasmids pPTH-AA and pPTH-AA-Eco (Tables 1 and 2), are considered to be rare degenerate codons in E. coli (Chen 1982). The more efficient PTH production by these two plasmids, as compared to others, contradicts earlier conclusions that tandem repeats of minor degenerate codons (Varenne 1986) and their proximity to the initiation codon would dramatically reduce the maximal level of protein synthesis (Chen 1990).

Comparison of the plasmids pPTH-AA, pPTH-CompB, pPTH-AA-Eco, pPTH-CompE-Eco, pPTH-GG and pPTH-GG-Eco with the other plasmids less efficient in PTH expression (Tables 1 and 2), indicates that the efficiency of expression might

be determined by the nucleotide (adenine, and likely to a small extent, guanine) composition of the amino-terminal coding sequence.

EXAMPLE 10: Characterization of PTH-(8-84) expressed by E. coli strain Y1091, transformed by pPTH-GG-Eco and other plasmids.

A series of new PTH genes were constructed with different previously constructed N-terminal (1-28) coding sequence truncated to the PTH-(29-84) coding sequence dominated by degenerate codons in the usage frequency favoured by E. coli. This was easily accomplished by ligating the PstI/HindIII-cut PTH-(29-84) coding sequence of pPTH-AA-Eco, which was isolated after PstI and HindIII restriction, to any of the PstI/HindIII-linearized plasmids described earlier (e.g. pPTH-wA, pPTH-GG and pPTH-hA, etc.). This would demonstrate the combined effect of E. coli-favoured codons in the mid/carboxyl section and host substitution.

E. coli strain Y1091 clone transformed by plasmid pPTH-GG-Eco, with G-rich codons at the sites coding for amino acids 1 through 4 yielded predominently the short fragment PTH-(8-84), with the yield of immunoreactive PTH increased to 25 mg/L or 3% of bacterial protein. These results demonstrate that potential ribosome binding sites upstream from codon ATG at position 8 enhance its misreading as a start codon.

Gel electrophoresis of a lysate of clone Y1091:pPTH-GG-Eco indicated mainly a new polypeptide more mobile than the synthetic human PTH (Figure 8), and it was eventually identified as PTH-(8-84) (Born 1987b). Western blotting with anti-PTH-(69-84) antibodies revealed the immunoreactive product as predominently the short analog, mixed with some PTH-(1-84) (Figure 9A). Blotting with the anti-PTH-(1-17) antibodies showed a dramatic loss of immunoreactivity of PTH-(8-84) in this lysate (Figure 9B), due to the loss of amino acid residue (1-7). The same

immunoreactive mixture was also observed in transformant possessing plasmid pPTH-GG (Figure 9). These results have indicated that even the Allegro PTH RIA does not exclude PTH-(8-84) in the estimation of PTH.

Western blot of cell lysate from clones Y1091:pPTH-hA-Eco and JM103:pPTH-hA also showed a 2:1 mixture of intact PTH and PTH-(8-84). Figure 10 illustrates Western immunoblotting of PTH product expressed by plasmids pPTH-hA and pPTH-hA-Eco. The membrane was immunoblotted with antibody specific to PTH-(69-84). All samples were whole cell lysates. Molecular weight standards are on the left margin.

Lane a - Y1091:pPTH-GG-Eco, 1 μl culture.
Lane b - mixed lysates of Y1091:pPTH-GG-Eco and Y1091:pUC8, 1 and 60 μl respectively.
Lane c - Y1091:pPTH-hA-Eco, 56 μl.
Lane d - JM103:pPTH-hA, 50 μl.
Lane e - Y1091:pUC8, 60 μl.

Both plasmids pPTH-hA and pPTH-hA-Eco yielded a 2:1 mixture of intact PTH and PTH-(8-84) (Lanes c and d).

In another clone pPTH-GA3-Eco which had only one G-rich codon TCG for amino acid one and A-rich codons for amino acids 2 through 4, moderate yield (8 mg/L) of intact PTH was detected without formation of PTH-(8-84), thereby further establishing the essential role of an A-rich degenerate codon at the first amino acid for the efficient expression of intact PTH and the absence of an efficient ribosome-binding site in amino acids 2 to 4 in avoiding expression of PTH-(8-84) fragment.

Since GTG, CTG and TTG have been used as a minor starting codon in some genes, any TG dinucleotide sequence in the PTH-(1-7) region may constitute a starting triplet codon in any reading frame resulting in competing translation, and reduction of expression of PTH. The absence of any such potential start codon in the PTH-(1-7) region in clones pPTH-AA, pPTH-AA-Eco, and pPTH-GA3-Eco,

etc., may partially account for the successful expression of intact PTH by E. coli transformed with these plasmids.

EXAMPLE 11: Synthesis of PTH analog.

We also carried out tests designed to produce PTH having greater stability and longer half-life. Typically, intact human PTH contains glycine as the amino acid residue at position 38. We have designed and produced a "mutant" PTH which, instead, contains an aspartic acid at position 38. Since the biological activity of PTH is believed to be located in the region of amino acids 1 through 34, accordingly a modification of the amino acid at position 38 should not alter the biological activity of our synthesized PTH. It should, however, increase the stability of the polypeptide during production in E. coli and its half-life in clinical application because aspartic acid at position 38 strengthens the potentially weak glycine-38 linkage. We constructed a plasmid PTH-AA-Eco-ASP-38 with codon GAC for aspartic acid-38, substituting GGC for glycine-38 in pPTH-AA-Eco to express this mutant form of PTH. Expression of the mutant plasmid in E. coli Y1091 was consistently higher than that of pPTH-AA-Eco (30 milligrams per litre compared with 20 milligrams per litre). Moreover it demonstrated its survivablity or stability during PTH production in E. coli. The asp-38 mutant protein has the same mobility as synthetic intact PTH (from Bachem Ltd.) on SDS-polyacrylamide gel electrophoresis with Coomassie blue stain. The N-terminus was sequenced and confirmed identical to human PTH up to 40 amino acid residues, with the exception of an aspartic acid residue at position-38. The amino acid composition analysis (by %) was identical to the theoretical calculation.

Example 12: Extraction and purification of recombinant PTH.

After harvesting from culture medium by centrifugation, cells were sonicated (1 min, pulsed) at 4°C

in a mixture (1 ml/g) of 1 M HCl containing 1% (w/v) NaCl, and 1% (v/v) TFA (Rabbani 1988) and centrifuged. The cell debris was reextracted a second time in the same fashion. The two extracts were pooled. This acidic extraction of E. coli Y1091:pPTH-AA-Eco enriched PTH-(1-84) to 10% of the total protein (60% recovery) (Rabbani 1988). The extract was adjusted to pH 3.8 with sodium hydroxide, diluted with water (4:1), and applied to a HL 10/10 Mono S column (Pharmacia) (Figure 11A is a chromatogram of the extract on cation exchanger Mono S, with concentration of PTH (black dots) in collected 1 ml. fractions estimated.) The column was eluted with a gradient of 0-2 M NaCl in 50 mM formic acid (pH 3.8) and PTH was recovered at 55% as evaluated by RIA. Immunoreactive fractions were pooled and applied to a 1x25 cm C_{18} silica (10 μm) column (Vydac) (Rabbani 1988). It was then eluted with a 1% /min gradient of 0.1% TFA/acetonitrile in 0.1% TFA/water (Figure 11B is a chromatogram of subsequent HPLC purification on C_{18} silica, with PTH-containing peak (stipple) indicated. Immunoreactive fractions containing mainly intact PTH were combined and lyopholized to yield intact PTH as a homogeneous product. The HPLC on C_{18} silica, which was capable of separating intact PTH, the unprocessed fMet-PTH, and analog PTH-(8-84) in a gradient of acetonitrile in 0.1% TFA (Rabbani 1988), revealed intact PTH as the only PTH-moiety isolated. Its purity was also confirmed by both gel electrophoresis (Figure 8) and Western blots (Figure 9). From 2 liters of culture medium, 6 mg of the recombinant intact PTH was obtained after lyophilization, with an overall recovery of 15%.

EXAMPLE 13: Characterization of purified intact PTH.

(a) Amino Acid Composition Analysis - Amino acid composition analyses of protein and of purified peptides were performed with a Durrum D-500 Analyzer. Samples (100 μg) were hydrolyzed in vacuo at 110°C in 6 N HCl for 24, 48 and 72 h and the data extrapolated to 0 h to correct

for hydrolytic losses. Tryptophan was determined following hydrolysis in 4 N methane sulphonic acid containing 0.2% 3-(2-aminoethyl)indole at 110°C for 20 h in vacuo (Simpson 1976). The combined cystine and cysteine content was determined after oxidation to cysteic acid (Hirs 1967) a hydrolysis in 6 N HCl at 110°C for 24 h.

Amino acid composition of the purified intact PTH was identical to the expected value for human PTH-(1-84) as summarized in Table 7 (Hendy 1981). In repeated analyses, values of 1.95, 1.91, 2.07 and 2.09 were obtained for the number of methionine residues after hydrolysis of 24 h (Table 7), thus generally consistent to the predicted value of 2 for the processed intact PTH (Hendy 1981). Methionine sulfoxide, which has been detected in some recombinant polypeptides secreted by E. coli (Hartmanis 1989), was not observed in the hydrolysed residues of PTH. Our data thus ruled out the presence of any significant amount of the oxidized Met-8, Met-18, or the unprocessed fMet.

For the production of the fully processed intact PTH, Ser-1, the residue adjacent to fMet, has a small radius of gyration essential for the efficient removal of fMet residue from the nascent polypeptide (Sherman 1985; Levitt 1976.

(b) Amino Acid Sequencing - PTH samples (500 pmole) on polyvinylidene difluoride membrane were analysed via gas-phase sequencing (Matsudaira 1987). Sequencing analysis of the purified intact PTH confirmed that the 40 residues at its amino-terminus was identical to that of human PTH-(1-84) (Hendy 1981). For sequencing other regions, the recombinant intact PTH was initially digested with endoproteinase Asp-N (Boehringer Mannheim), which has been reported to cleave specifically at the amino-terminus of the aspartic acid residues. The resulting peptide mixture was separated by HPLC on C_{18} silica using a 1% /min gradient of acetonitrile in 0.1 % TFA/water. After sequencing, one short peptide revealed a sequence of 11 amino acid residues identical to the PTH-(74-84) terminus

(Hendy 1981). The amino acid sequences of other analogs PTH-(8-84) and PTH-(3-84) were also established by the same analytical method.

(c) HyperMass Molecular Weight Determinations - IonSpray mass spectra of the purified recombinant PTH-(1-84) and the PTH-(8-84) analog were obtained by the API III System (SCIEX, Mississauga, ONT). Ionspray mass spectra of recombinant PTH-(1-84) (Figure 12A) and analog PTH-(8-84) (Figure 12B) predominantly showed peaks of the molecular ions possessing different numbers of positive charge (H+). Based on the m/z value (i.e. mass/charge) and the charge number (z, indicated in parenthesis) of individual peaks, the molecular mass was calculated by the formula of (m/z x z) - z in four most prominent peaks. In the spectrum of PTH-(1-84) (Figure 12A), molecular mass of 9424.90, 9425.91, 9426.91 and 9424.92 Daltons was obtained, with an average of 9425.66. In the spectrum of PTH-(8-84) (Figure 12B), molecular mass of 8668.30, 8668.71, 8669.01 and 8666.92 Daltons was calculated, with an average of 8668.73. The experimental molecular masses of 9425.66 Daltons for PTH-(1-84) and 8668.73 Daltons for PTH-(8-84), were thus consistent to their respective theoretical values of 9425.26 and 8668.36 Daltons. The absence of other unidentified ions in the mass spectrum (Figure 12A) of PTH-(1-84) generally confirmed the purity of this sample.

(d) Bioassay - With the identity and purity established, recombinant intact PTH (open circles) was compared with synthetic intact PTH (solid squares) (manufactured by Bachem Corp., Torrance, California) in an adenylate cyclase bioassay (Figure 13) (Rabbani 1988). This test involved the stimulation of adenylate cyclase by the PTH samples at different concentrations, to produce cyclic AMP in osteosarcoma cells (UMR 106). Cyclic AMP is a secondary messenger in the functional pathway of this hormone. Our experimental results indicated average k_{act} values (PTH concentration for achieving half maximal stimulation) of 1.6 nM (k_{act} range 1.5- 1.7

nM) for our recombinant intact PTH, and 3.8 nM (range 3.5-4.2 nM) for the synthetic intact PTH (Figure 13). In comparison, PTH-(1-84) samples previously prepared by various recombinant DNA methods (Breyel 1984; Hogset 1990; Rabbani 1988; Wingender 1989), have been reported to have k_{act} values equal to the synthetic PTH-(1-84) standard. The small k_{act} (1.6 nM) of our recombinant PTH-(1-84), which was half the k_{act} (3.8 nM) of the synthetic standard, indicated our recombinant sample being the most bioactive of any PTH-(1-84) sample reported to date, synthetic or recombinant DNA-derived. Its strong bioactivity also indirectly confirmed its integrity. Analogs, such as the nascent fMet-PTH and the methionine-oxidized intact PTH, if present in significant amount, would have reduced the bioactivity of the recombinant PTH since both analogs have very weak bioactivity (Rabbani 1988; Tashjian 1964).

EXAMPLE 14: Adenine-rich Nucleotide Sequence at the N-terminal Region Enhanced Expression of Analog PTH-(3-84)

The strategy of using an adenine-rich nucleotide sequence in the N-terminal domain to enhance gene expression was also demonstrated in the case of PTH-(3-84) since it and other fragments of PTH may demonstrate biological activity. The short analog PTH-(3-84) has previously been produced as a minor byproduct during the expression of the preproPTH cDNA in E. coli. For improving production of this analog, a plasmid pPTH-(3-84)-AA-Eco was designed to possess nucleotide sequence identical to that of pPTH-AA-Eco, with the loss of the two codons TCA.GTA for residues Ser-1 and Val-2 of PTH. The new plasmid pPTH-(3-84)-AA-Eco was assembled in the same manner as the latter plasmid. Expression in strain Y1091 yielded PTH-(3-84) as the only PTH product at 15 mg/L. This was confirmed by gel electrophoresis, Western immunoblotting and amino acid sequencing.

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Table 1

Expression of PTH genes possessing different amino-terminal nucleotide sequences in E. coli strain JM103.

PTH gene -containing plasmids	PTH[a] mg/L	N-terminal coding sequences[b] 1 Ser	2 Val	3 Ser	4 Glu	5 Ile
pPTH-AA	3.9	TCA	GTA	TCA	GAA	ATA
pPTH-CC	0.15	C	C	C		C
pPTH-GG	10[c]	G	G	G	G	
pPTH-TT	0.25	T	T	T		T
pPTH-CompB	1.1	AGT	T	AGT		T
pPTH-wA[d]	3.4					
pPTH-wxA[e]	3.5					
pPTH-hA	0.3[f]	T	G	AGT		
pPTH-84c[g]	0.19[f]	G	T	T	G	C

[a] Estimated by Allegro RIA.

[b] Nucleotide sequence encoding the PTH-(1-5) region of pPTH-AA is presented. For other plasmids, only nucleotides different from pPTH-AA are presented in this Table. Codon differences in other regions are stated individually.

[c] A 1:5 mixture of PTH-(1-84) and PTH-(8-84).

[d] GTA-21 and TTA-24.

[e] AAA-13, -26, -27, TTA-15, -24, TCA-17 and GTA-21.

[f] A 2:1 mixture of PTH-(1-84) and PTH-(8-84).

[g] GTG-8, AAC-10 and TTG-11; see Preliminary Example.

Table 2

Expression of PTH genes possessing different amino-terminal nucleotide sequences in E. coli strain Y1091.

PTH gene -containing plasmids	PTH[a] mg/L (% protein)	N-terminal coding sequences[b] 1 Ser	2 Val	3 Ser	4 Glu	5 Ile
pPTH-AA-Eco	20 (2.5)	TCA	GTA	TCA	GAA	ATA
pPTH-wA-Eco[c]	15 (2)					
pPTH-A-Eco(18-84)[d]	14					
pPTH-A-Eco(8-84)[e]	7					
pPTH-CompE-Eco	12	AGC		AGC		
pPTH-GG-Eco	25[f]	G	G	G	G	
pPTH-hA-Eco	0.3[g]	T	G	AGT		

[a] Estimated by Allegro RIA. Values in parenthesis ( ) are yields as percentage of bacterial protein, calculated by integrating the areas under the peak after densitometric scanning of gel.

[b] Nucleotide sequence encoding the PTH-(1-5) region of pPTH-AA-Eco is presented. For other plasmids, only nucleotides different from pPTH-AA-Eco are presented in this Table. Codon differences in other regions are stated individually.

[c] GTA-21 and TTA-24.

[d] CGT-20, -25, CTG-24 and AAA-27.

[e] AAC-10, CTG-11, -15, -24, AAA-13, -26, -27, CGT-20, GTG-21 and CGC-25.

[f] A 1:5 mixture of PTH-(1-84) and PTH-(8-84).

[g] A 2:1 mixture of PTH-(1-84) and PTH-(8-84).

Table 3

Effect of potential ribosome-binding site (underlined) in N-terminal coding region of different plasmids on the expression of intact PTH and PTH-(8-84) in E. coli Y1091.

Clone	PTH yield mg/L intact	(8-84)	N-terminal coding sequence 1 ser	2 val	3 ser	4 glu	5 ile	6 gln	7 leu	8 met
pPTH-AA-Eco	20		TCA	GTA	TCA	GAA	ATA	CAA	TTA	ATG
pPTH-CompE-Eco	12		AGC	GTA	AGC	GAA	ATA			
pPTH-GA3-Eco	8.1		TCG	GTA	TCA	GAA	ATA			
pPTH-GA10-Eco	5.9		TCA	GTG	TCA	GAA	ATA			
pPTH-GA1-Eco	5.5		TCG	GTG	TCA	GAA	ATA			
pPTH-CC-Eco	0.3		TCC	GTC	TCC	GAA	ATC			
pPTH-TT-Eco	0.2		TCT	GTT	TCT	GAA	ATT			
pPTH-GA8-Eco	4.5	1.5	TCA	GTA	TCA	GAG	ATA			
pPTH-GA9-Eco	4.0	1.2	TCA	GTG	TCA	GAG	ATA			
pPTH-GA4-Eco	3.5	1.5	TCG	GTG	TCA	GAG	ATA			
pPTH-GA5-Eco	2.5	1	TCG	GTG	TCG	GAA	ATA			
pPTH-hA-Eco	0.2	0.1	TCT	GTG	AGT	GAA	ATA			
pPTH-GA6-Eco	4	20	TCA	GTA	TCG	GAG	ATA			
pPTH-GG-Eco	4	21	TCG	GTG	TCG	GAG	ATA			
pPTH-GA11-Eco	1	23	TCT	GTC	TCG	GAG	ATA			
pPTH-GA12-Eco		15	TCT	GTT	TCG	GAG	ATA			

_____ weak ribosome-binding site.

═══ strong ribosome-binding site.

Table 4

Expression of plasmids pPTH-AA, pPTH-wA and pPTH-wxA in E. coli strains JM103 and Y1091.

Plasmids	JM103		Y1091	
	Intact PTH* (mg/L)	% bacterial protein	Intact PTH* (mg/L)	% bacterial protein
pPTH-AA	3.9	0.5	6.5	0.8
pPTH-wA	3.4	0.4	7.0	0.9
pPTH-wxA	3.5	0.4	6.5	0.8

*Estimated by the Allegro PTH Radioimmunoassay

Table 5

Expression of PTH gene (plasmid pPTH-AA-Eco)
in different E. coli strains JM103, HB101 and Y1091.

Culture Conditions	JM103		HB101		Y1091	
	Intact PTH* (mg/L)	% bacterial protein	Intact PTH* (mg/L)	% bacterial protein	Intact PTH* (mg/L)	% bacterial protein
8 hr [-IPTG]	0	0	7.1	0.9	20.0	2.5
8 hr [+IPTG]	5.5	0.7	6.1	0.8	19.0	2.3
16 hr [-IPTG]	0	0	1.7	0.02	0	0
16 hr [+IPTG]	4.6	0.6	3.9	0.4	3.3	0.04

*Estimated by the Allegro PTH Radioimmunoassay

Table 6

Production of PTH in E. coli Y1091: pPTH-AA-Eco, in relationship to culture time.

Culture Time (hr.)	Intact PTH* (mg/L)	% bacterial protein
5	5.5	0.7
6	10.5	1.3
8	17.1	2.1
10	20.0	2.5
18	3.0	0.4

*Estimated by the Allegro PTH Radioimmunoassay.
Cells grown in 2YT medium without induction by IPTG.

Table 7

Amino acid composition of the purified recombinant intact PTH.

Amino acid	Residues/mol determined 24h	48h	72h	nearest integer	pTH-(1-84)
Asx	10.00	10.00	10.00	10[a]	10
Thr	0.82	0.82	0.80	1	1
Ser	6.08	5.46	4.90	7[b]	7
Glx	11.20	11.20	11.23	11	11
Pro	3.00	2.89	2.87	3	3
Gly	3.94	3.98	3.90	4	4
Ala	7.00	6.99	7.03	7	7
Cys	0.00			0	0
Val	7.97	8.04	7.86	8	8
Met	1.95[c]	1.86	1.80	2	2
Ile	0.98	0.98	0.95	1	1
Leu	9.77	9.75	9.62	10	10
Tyr	0.00	0.00	0.00	0	0
Phe	0.99	1.04	1.03	1	1
His	4.04	4.09	4.06	4	4
Lys	9.07	9.04	9.01	9	9
Arg	4.95	5.03	4.97	5	5
Trp	1.06			1	1
Total				84	84

[a] 10 Asx residues/mol is assumed.

[b] Extrapolated value of 6.7 at zero time.

[c] Values of 1.91, 2.07 and 2.09 obtained in repeated analyses.

CLAIMS:

1. A synthetic nucleotide sequence encoding intact human parathyroid hormone or a biologically active analog wherein at least some codons in the N-terminal region are selected from adenine-rich degenerate codons.

2. The nucleotide sequence according to claim 1 wherein codons encoding amino acids 1 through 28 are selected from adenine-rich degenerate codons available for coding such amino acids.

3. The nucleotide sequence according to claim 1 wherein codons encoding amino acids 1 through 5 are selected from adenine-rich degenerate codons available for coding such amino acids.

4. The nucleotide sequence according to claim 1 wherein codons encoding amino acids 1 through 3 are selected from adenine-rich degenerate codons available for coding such amino acids.

5. The nucleotide sequence according to claim 1 wherein codons encoding amino acids 3 through 5 are selected from adenine-rich degenerate codons available for coding such amino acids.

6. The nucleotide sequence according to claim 1 wherein codons encoding amino acids 2 and 3 are selected from adenine-rich degenerate codons available for coding such amino acids.

7. The nucleotide sequence according to claim 1 wherein the degenerate codons encoding amino acids 1 through 5 are TCA, GTA, TCA, GAA and ATA respectively.

8. The nucleotide sequence according to claim 1 wherein the degenerate codons encoding amino acids 1 through 3 are TCA, GTA and TCA respectively.

9. The nucleotide sequence according to claim 1 wherein the degenerate codons encoding amino acids 1 through 3 are TCG, GTA, and TCA respectively.

10. The nucleotide sequence according to claim 1 wherein the degenerate codons encoding amino acids 1 through 7 are selected so as to avoid coding for a potential ribosome-binding site.

11. The nucleotide sequence according to claim 1 wherein at least some of the codons encoding amino acids 29 through 84 are degenerate codons in the usage frequency favoured by Escherichia coli.

12. The nucleotide sequence according to claim 1 wherein at least some of the codons encoding amino acids 29 through 84 are degenerate codons in the usage frequency favoured by yeast.

13. The nucleotide sequence according to claim 1 wherein the codon encoding the amino acid 38 is selected so as to encode aspartic acid.

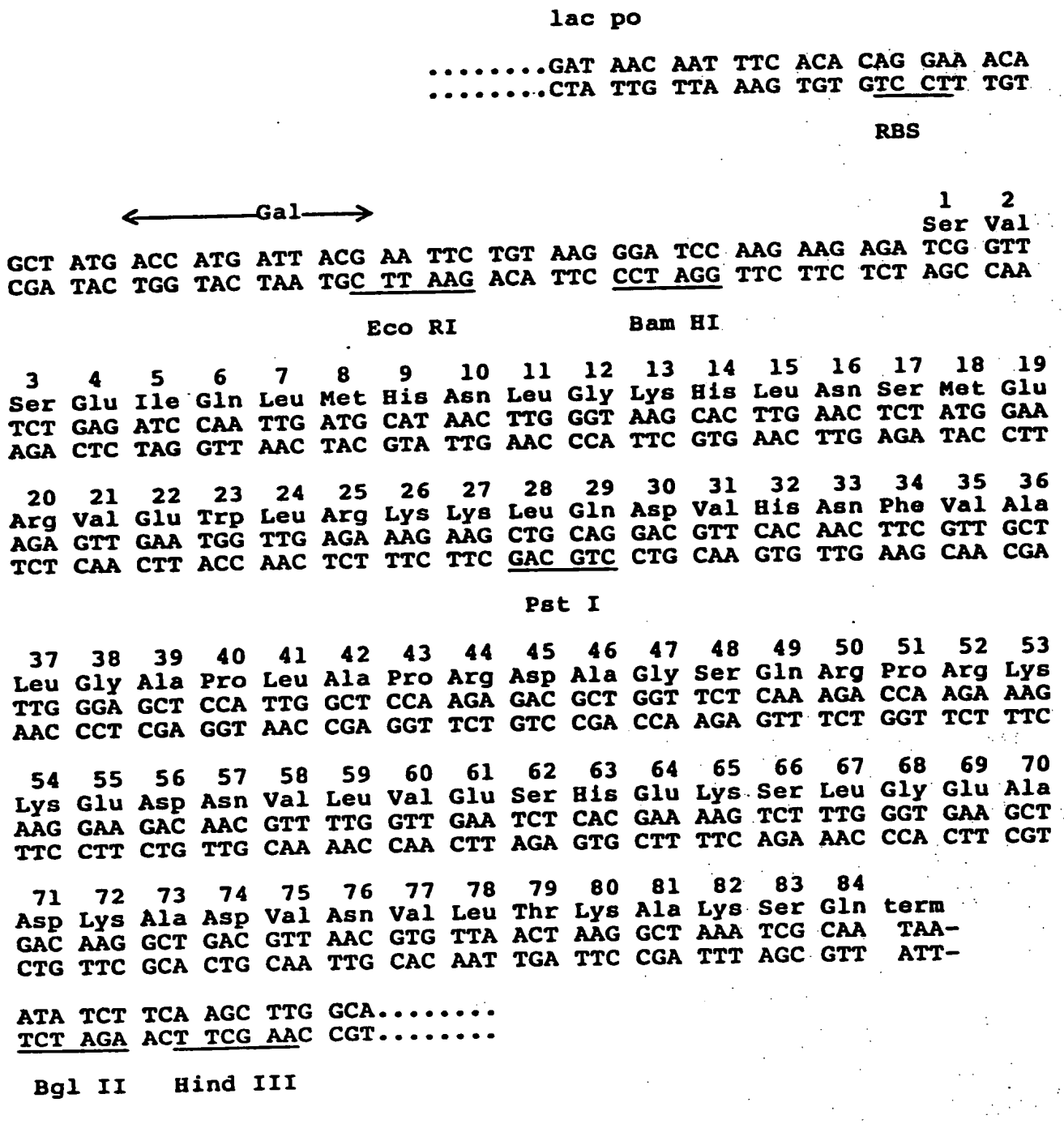
14. A plasmid containing the synthetic nucleotide sequence according to any one of claims 1 to 13.

15. An expression system comprising a microorganism transformed by the plasmid according to claim 14.

16. An expression system according to claim 15 wherein the microorganism is E. coli.

17. An expression system according to claim 15 wherein the microorganism is E. coli Y1091 (lon).

18. A method of obtaining intact human parathyroid hormone or a biologically active analog comprising transforming a microorganism with the plasmid of claim 14, and expressing and recovering intact human parathyroid hormone or a biologically active analog.

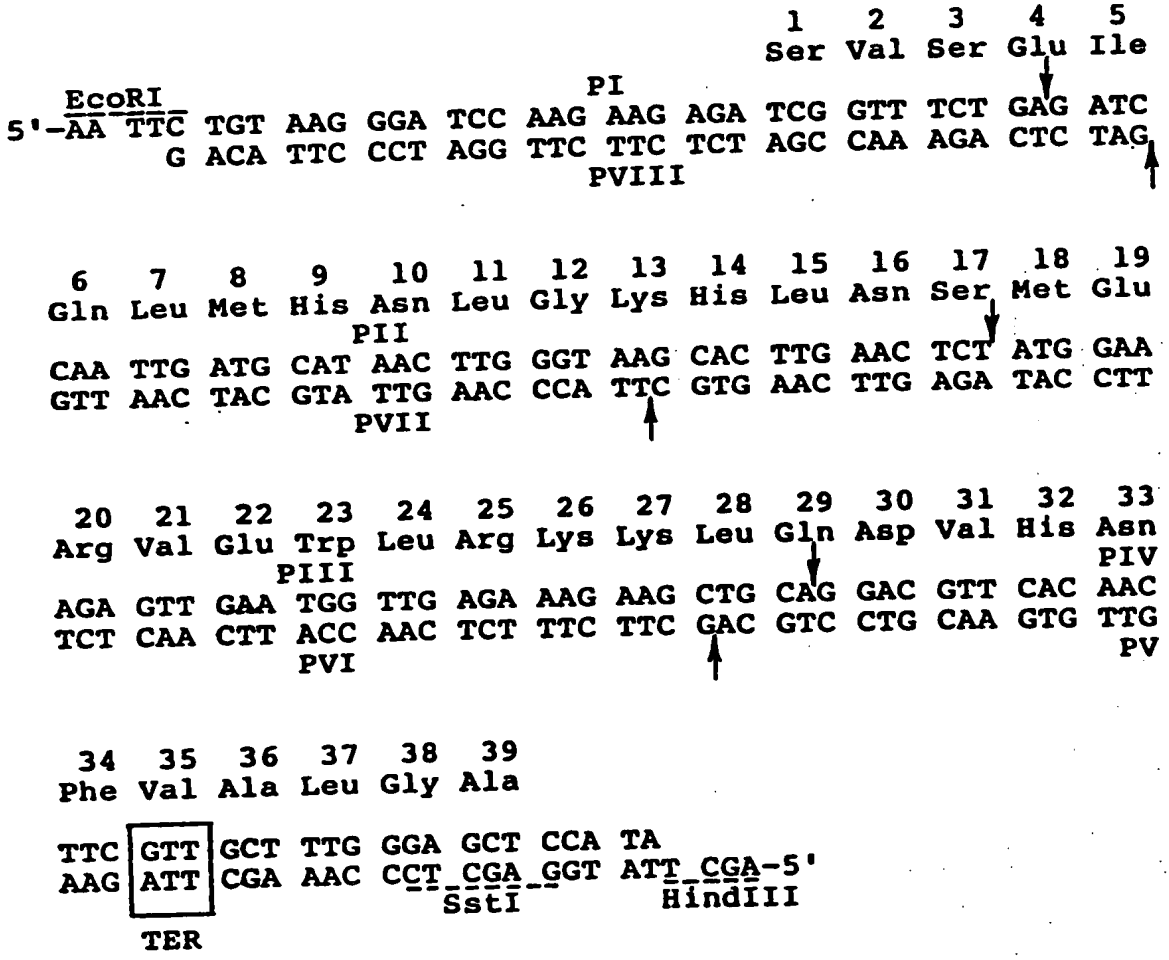


NUCLEOTIDE SEQUENCE ENCODING PTH IN PLASMID pPTH-84

FIG.1

SUBSTITUTE SHEET

A



DESIGN OF DNA SEQUENCES ENCODING PTH AND ANALOGS. ENDS OF OLIGONUCLEOTIDES ARE INDICATED BY ARROWS. BASE-MISMATCHING REGIONS FOR GENERATING ANALOGS ARE CONTAINED IN BOXES.
(A) OLIGONUCLEOTIDES PI-PVIII CODING FOR PTH (POSITIONS 1-40) FOR THE CONSTRUCTION OF PLASMIDS pPTH-34 AND pPTH-40.

FIG. 2

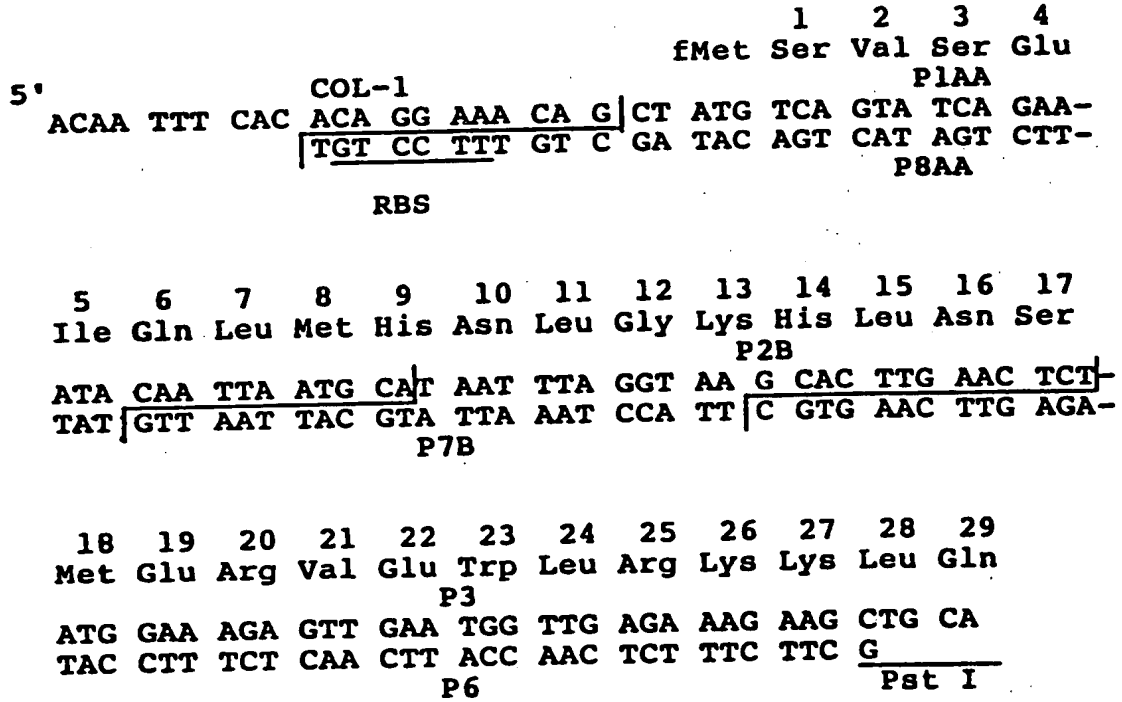
..... CONTINUED

B

```
          40  41  42  43  44  45  46  47  48  49  50
        Pro Leu Ala Pro Arg Asp Ala Gly Ser Gln Arg
                            PIX
   SstI                                 ↓
        CCA TTG GCT CCA AGA GAC GCT GGT TCT CAA AGA
3'-T CGA GGT AAC CGA GGT TCT CTG CGA CCA AGA GTT TCT
                          PXIV

 51  52  53  54  55  56  57  58  59  60  61  62  63
Pro Arg Lys Lys Glu Asp Asn Val Leu Val Glu Ser His
            PX                          ↓
CCA AGA AAG AAG GAA GAC AAC GTT TTG GTT GAA TCT CAC
GGT TCT TTC TTC CTT CTG TTG CAA AAC CAA CTT AGA GTG
↑           PXV

 64  65  66  67  68  69  70  71  72  73  74  75  76
Glu Lys Ser Leu Gly Glu Ala Asp Lys Ala Asp Val Asn
                        PXI                     ↓
GAA AAG TCT TTG GGT GAA GCT GAC AAG GCT GAC GTT AAC
CTT TTC AGA AAC CCA CTT CGA CTG TTC CGA CTG CAA TTG
↑                       PXIV

 77  78  79  80  81  82  83  84
Val Leu Thr Lys Ala Lys Ser Gln Ter
        PXII
GTG TTA ACT AAG GCT AAA TCG CAA [TAA] AGA TCT TGA
CAC AAT TGA TTC CGA TTT AGC GTT [ACA] TCT AGA ACT TCG A-5'
      ↑  PXIII                      BglII    HindIII
                                   CYS
```

(B) OLIGONUCLEOTIDES PIX-PXVI CODING FOR PTH (POSITIONS 38-84) FOR THE CONSTRUCTION OF PLASMIDS pPTH-84 AND pPTH-87.

FIG. 2 (CONTINUED)



OLIGONUCLEOTIDES COL-1, P1AA, P2B, P3, P6, P7B AND P8AA FOR THE CONSTRUCTION OF ADENINE-RICH CODING SEQUENCE OF PTH-(1-28) IN PLASMID pPTH-AA.

FIG. 3

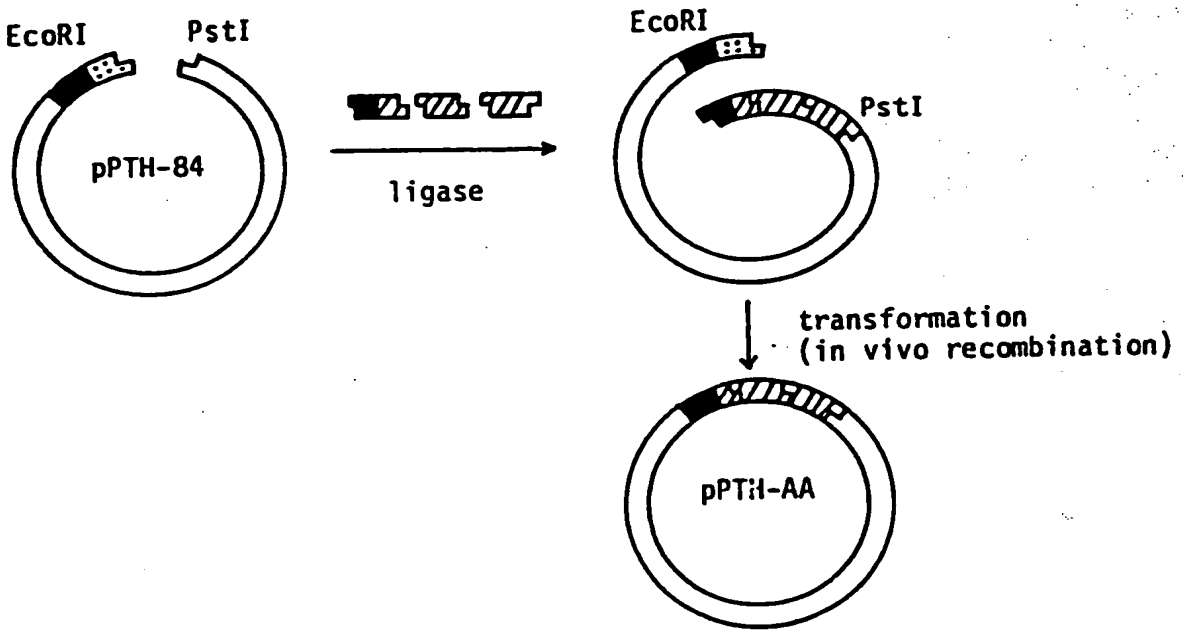
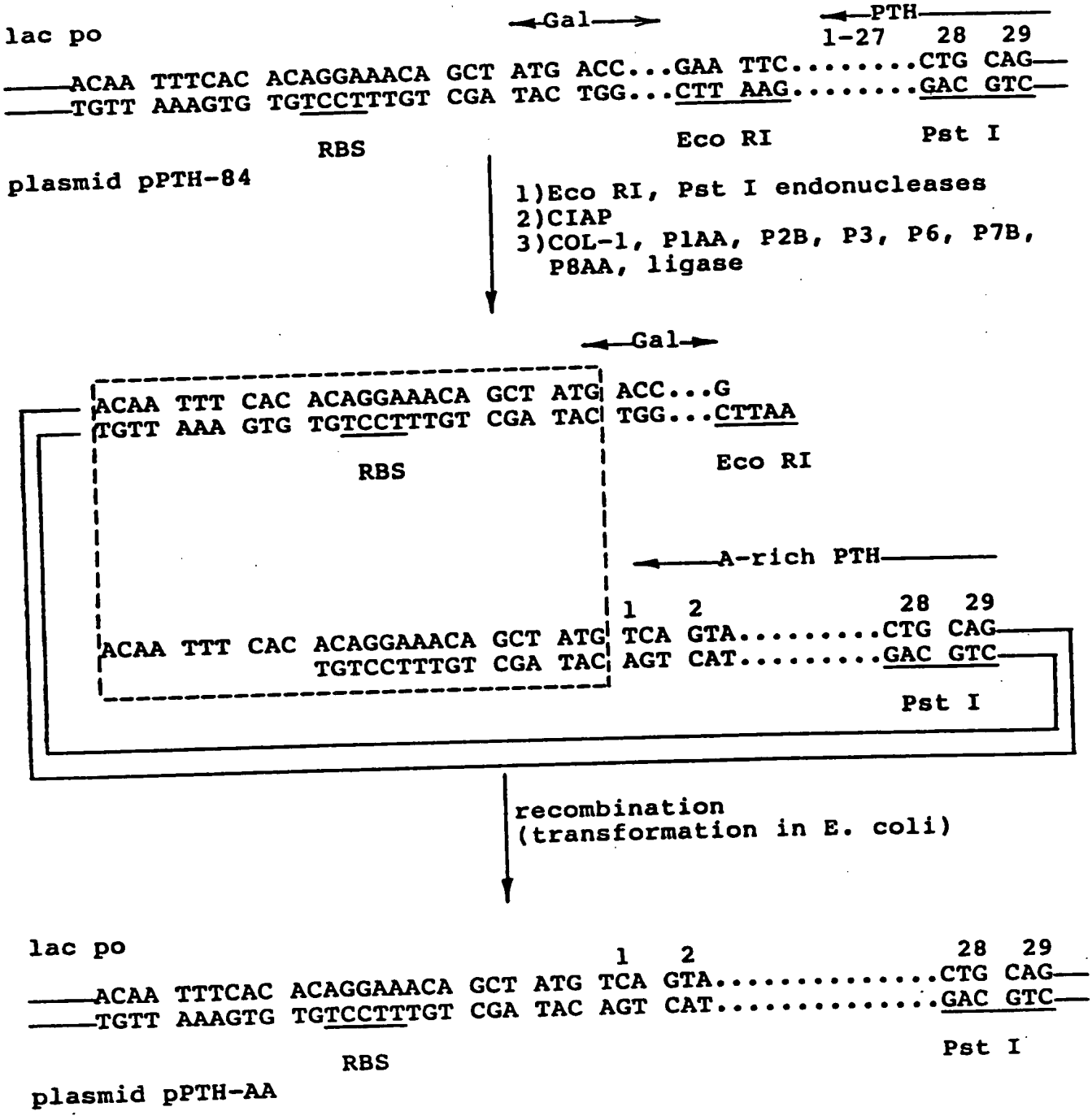

EcoRI
PstI
pPTH-84
ligase
EcoRI
PstI
transformation
(in vivo recombination)
pPTH-AA

FIG. 4



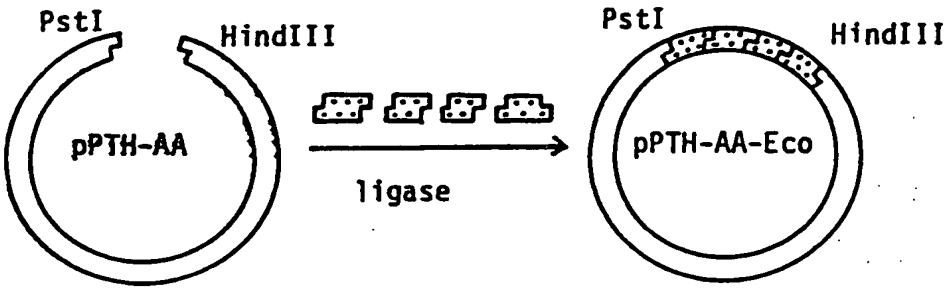
SYNTHESIS OF PLASMID pPTH-AA CONTAINING AN A-RICH PTH-CODING SEQUENCE

FIG. 5

```
 28  29  30  31  32  33  34  35  36  37  38  39  40   41
Leu Gln Asp Val His Asn Phe Val Ala Leu Gly Ala Pro  Leu
                                  P103b
      G GAC GTT CAC AAT TTC GTT GCG CTG GGC GCT CCG CT  G-
 AC GTC CTG CAA GTG TTA AAG CAA CGC GAC CCG CGA GGC GA  C-
          P204a
  Pst I

 42  43  44  45  46  47  48  49  50  51  52  53  54  55
Ala Pro Arg Asp Ala Gly Ser Gln Arg Pro Arg Lys Lys Glu
                        P104
GCA CCG CGT GAC GCT GGT TCT CAA CGC CCG CGT AAG AAA GAA-
CGT GGC GCA CTG CGA CCA AGA GTT GCG GGC GCA TTC TTT CTT-
                    P203

 56  57  58  59  60  61  62  63  64  65  66  67  68  69  70
Asp Asn Val Leu Val Glu Ser His Glu Lys Ser Leu Gly Glu Ala
                                    P105
GAT AAC GTT CTG GTT GAA TCC CAT GAG AAA TCT CTG GGC GAA GCC-
CTA TTG CAA GAC CAA CTT AGG GTA CTC TTT AGA GAC CCG CTT CGG-
                                P202

 71  72  73  74  75  76  77  78  79  80  81  82  83  84
Asp Lys Ala Asp Val Asn Val Leu Thr Lys Ala Lys Ser Gln Term
                                            P106
GAC AAA GCG GAT GTG AAC GTT CTG ACC AAA GCT AAA TCC CAG  TAA-
CTG TTT CGC CTA CAC TTG CAA GAC TGG TTT CGA TTT AGG GCT  ATT-
                                P201

-AGA TCT TGA
-TCT AGA ACT TCG A

        Hind III
```

OLIGONUCLEOTIDES P103b, P104, P105, P106, P201, P202, P203 and P204a FOR THE CONSTRUCTION OF NUCLEOTIDE SEQUENCE ENCODING PTH-(29-84) with E. COLI-FAVORED CODONS.

FIG.6



DIAGRAMMATIC SCHEME FOR THE CONSTRUCTION OF pPTH-AA-Eco. OVERLAPPING OLIGONUCLEOTIDE DUPLEXES P103b, P104, P105, P106, P201, P202, P203, AND P204a (STIPPLED), WHICH CONSTITUTED THE CODING SEQUENCE FOR PTH-(29-84) WERE LIGATED TO THE PstI/HindIII-LINEARIZED PLASMID TO YIELD A NEW PLASMID pPTH-AA-Eco.

FIG. 7

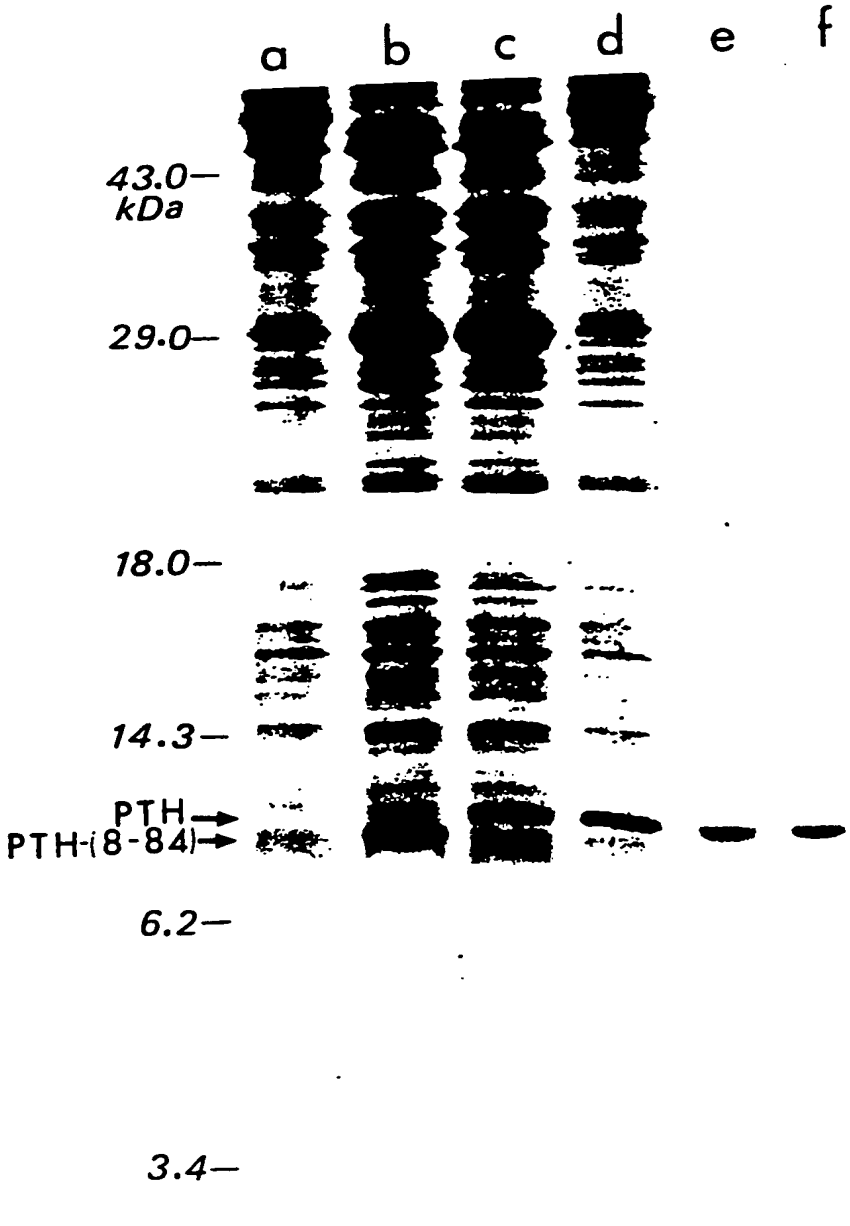


FIG. 8

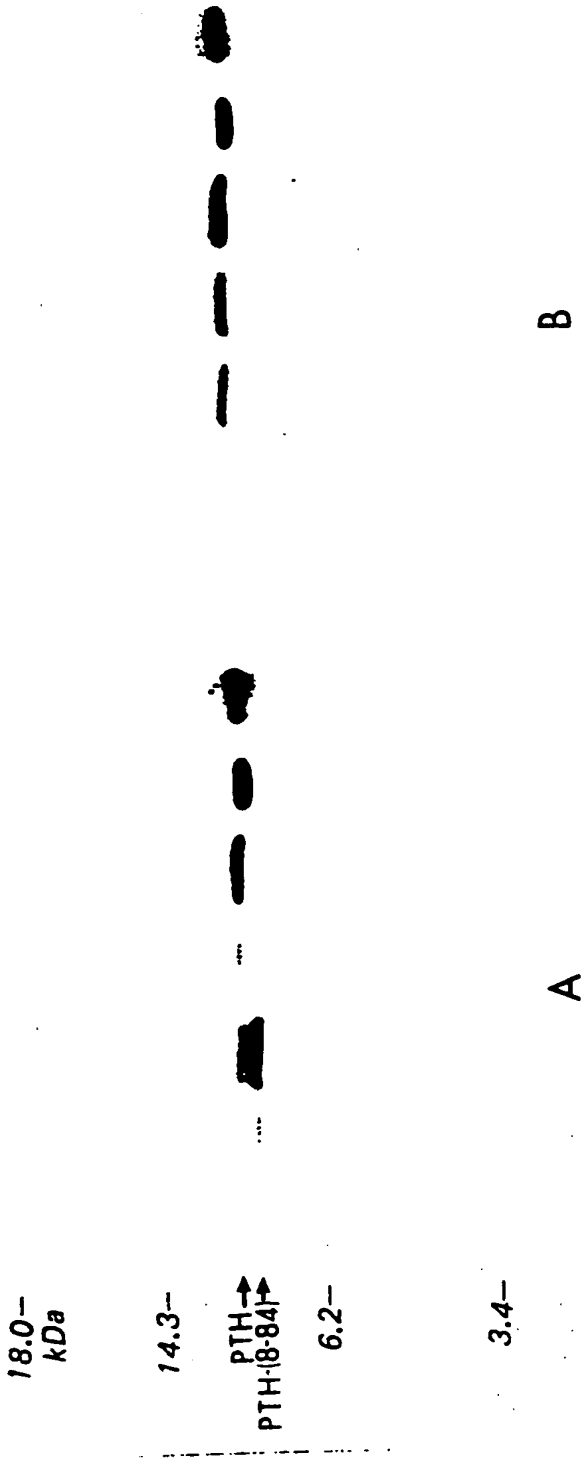


FIG. 9

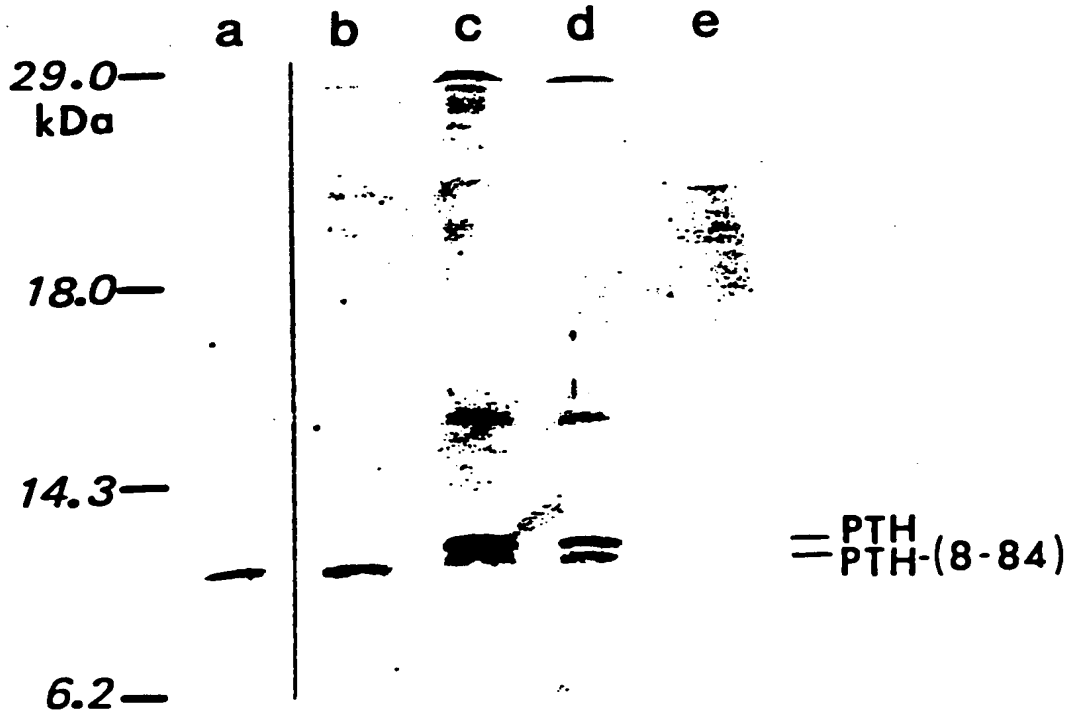


FIG. 10

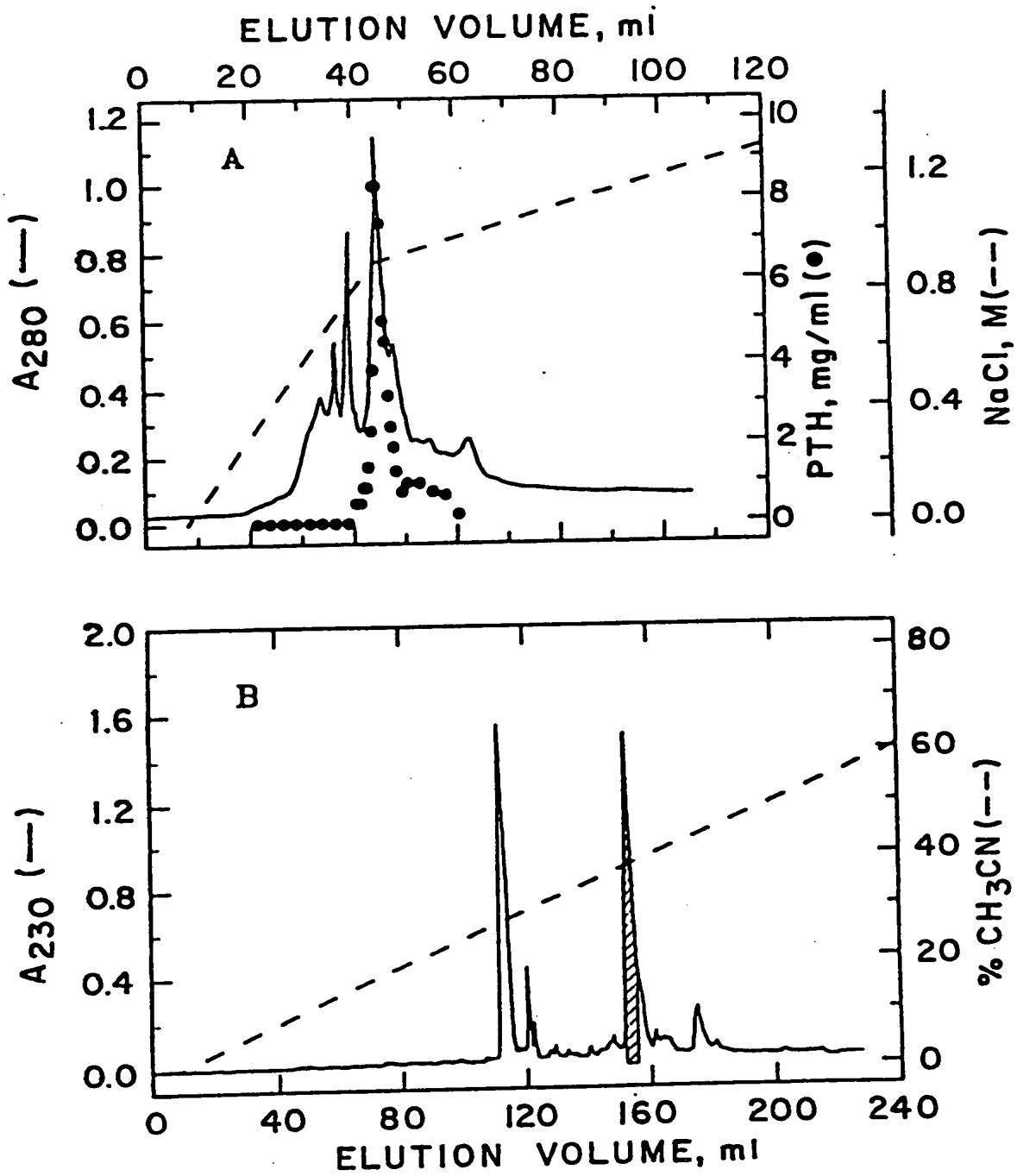

ELUTION VOLUME, ml
0 20 40 60 80 100 120
A
A280 (—)
PTH, mg/ml (●)
NaCl, M(---)
B
A230 (—)
% CH3CN(---)
0 40 80 120 160 200 240
ELUTION VOLUME, ml

FIG. 11

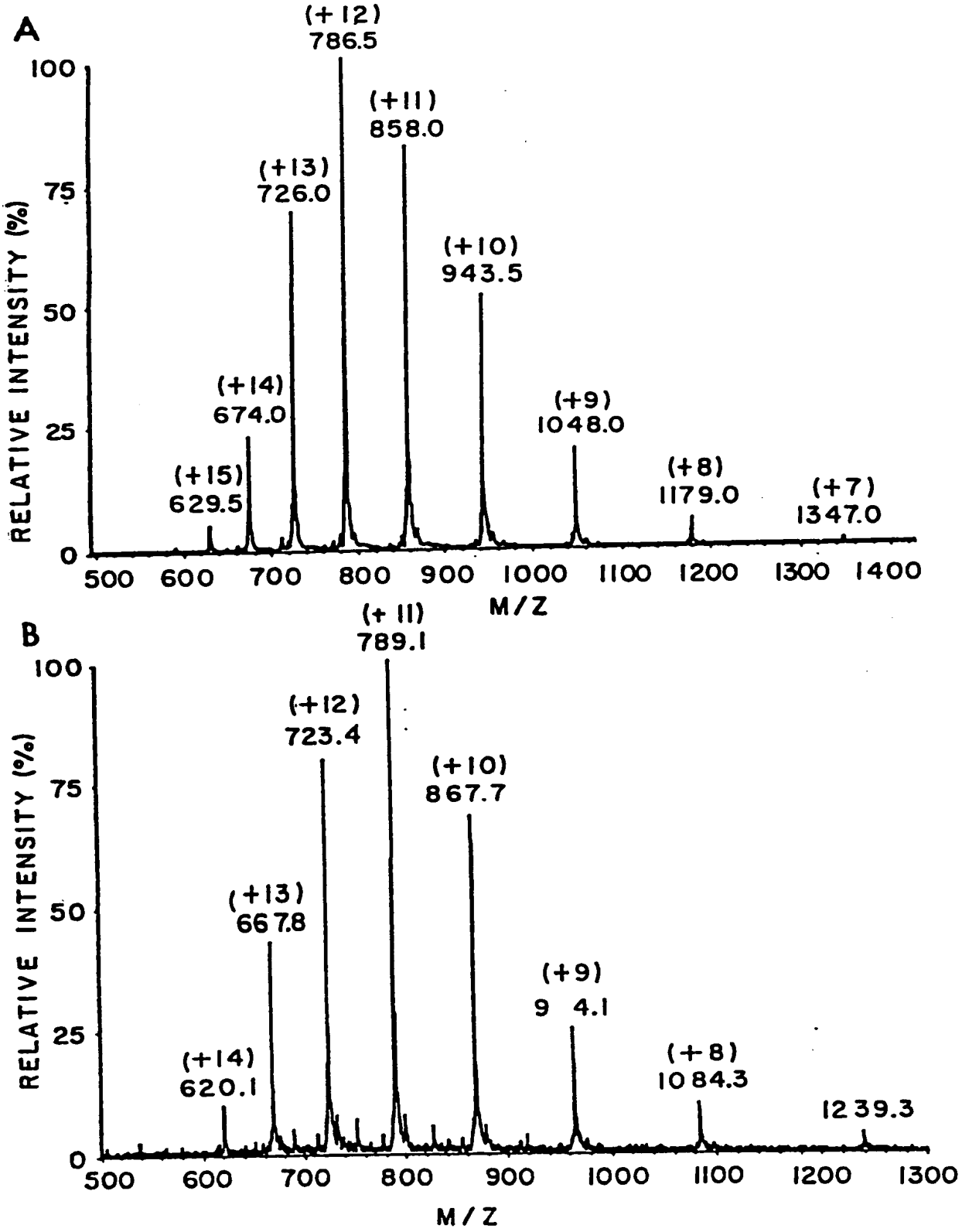
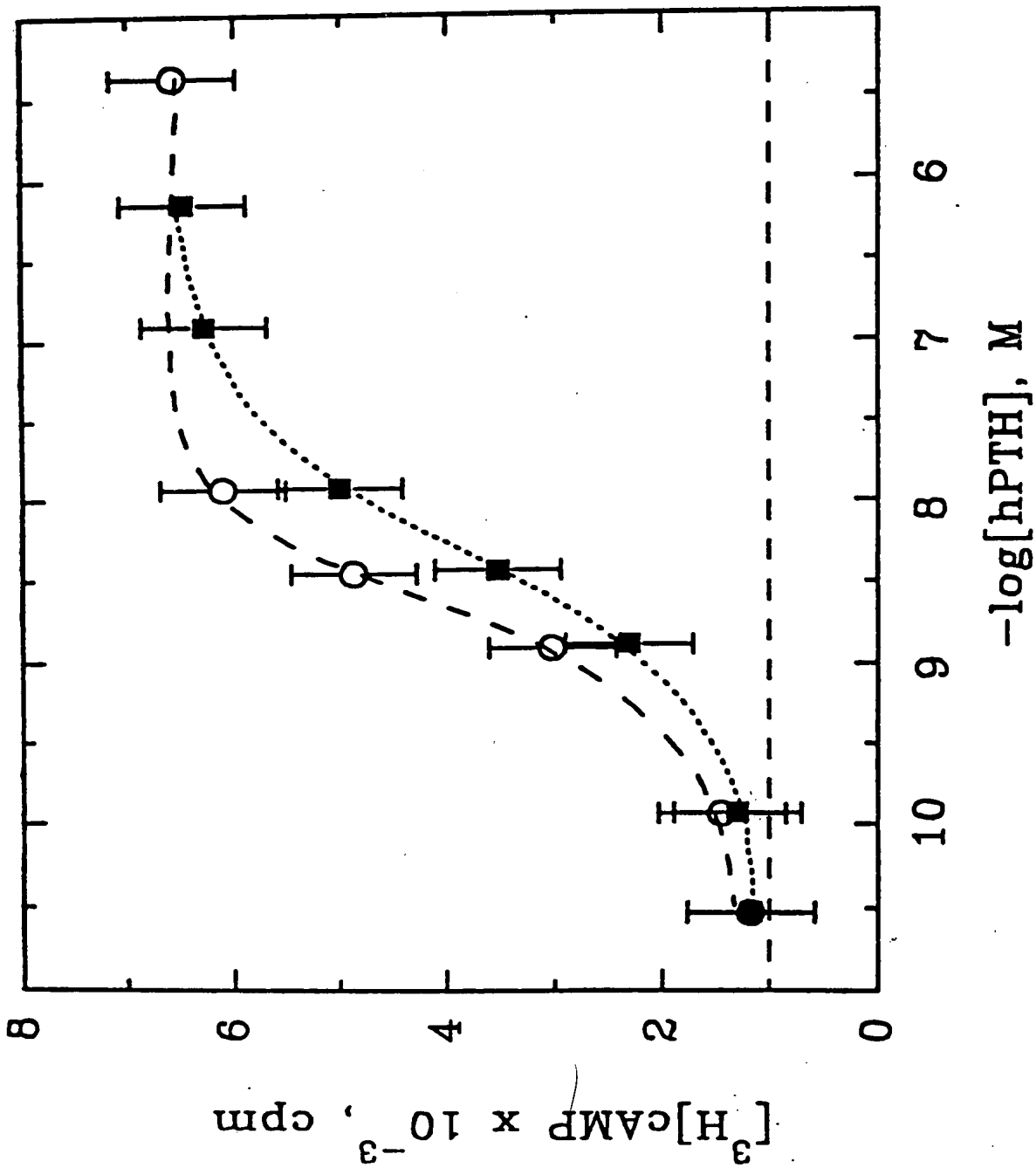

A
(+12)
786.5
(+11)
858.0
(+13)
726.0
(+10)
943.5
(+14)
674.0
(+9)
1048.0
(+15)
629.5
(+8)
1179.0
(+7)
1347.0
RELATIVE INTENSITY (%)
100
75
50
25
0
500 600 700 800 900 1000 1100 1200 1300 1400
M/Z
B
(+11)
789.1
(+12)
723.4
(+10)
867.7
(+13)
667.8
(+9)
9 4.1
(+14)
620.1
(+8)
1084.3
1239.3
RELATIVE INTENSITY (%)
100
75
50
25
0
500 600 700 800 900 1000 1100 1200 1300
M/Z

FIG. 12

FIG.13



INTERNATIONAL SEARCH REPORT

International Application No PCT/CA 90/00335

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 N 15/67, C 12 N 1/21 //(C 12 N 1/21, C 12 R 1:19) C 12 N 15/16,

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 07 K, C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	European Congress of Biotechnology, volume 3, 1984, E. Breyel et al,: "Synthesis of mature human parathyroid hormone in Escherichia coli", pages 363-369 see the whole document cited in the application	1
A	WO, A, 88/03165 (G. MODIANO et al.) 5 May 1988 see the whole document	1
A	Biochimica et Biophysica Acta, volume 950, 1988, Elsevier, (Amsterdam, NL), G. Morelle et al: "Increased synthesis of human parathyroid hormone in Escherichia coli through alterations of the 5' untranslated region", pages 459-462 see the whole document cited in the application	1

* Special categories of cited documents: [14]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14th January 1991	- 1. 02. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. PEIS

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. CA 9000335 SA 40446

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 28/01/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8803165	05-05-88	AU-A- 8104887	25-05-88
		EP-A- 0383751	29-08-90
		JP-T- 2501108	19-04-90